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DER GEGENSEITIGE ANTAGONISMUS DER STRAHLENPILZE, I. UNTERSUCHUNG DES ACTINOMYCES-STAMMES M 17

Von

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Über den gegenseitigen Antagonismus der Strahlenpilze (Aktinomyzeten) sind bisher nur wenige Angaben veröffentlicht worden, obgleich mit solchen Wirkungen in dieser Gruppe der sich hinsichtlich ihrer ökologischen Ansprüche so nahestehenden Mikroorganismen — unter natürlichen Verhältnissen — sehr häufig zu rechnen ist.

Im Laufe unserer in den letzten Jahren durchgeführten Untersuchungen [1] wurden zahlreiche Aktinomyzetenstämme gefunden, deren auf künstlichen Nährböden beobachtete antibiotische Wirkung sich vor allem gegenüber anderen Strahlenpilzstämmen zeigte. Im nachstehenden soll nun über die Untersuchung eines dieser Strahlenpilze, über den von uns »Actinomyces M 17« genannten Stamm berichtet werden.

Material und Methoden

Glukose-Asparagin-Agar: Glukose 10 g, Asparagin 0,5 g, K_2HPO_4 0,5 g, Agar 15 g. dest. Wasser 1000 ml p_H 6,2—6,4.

Glyzerin-Natriumnitrat-Agar [4]: K_2HPO_4 0,1%, $MgSO_4$ 0,05%, KCl 0,05%, $FeSO_4$ 0,001%, $NaNO_3$ 0,2%, Glyzerin 3,0%, Agar 1,5%.

Möhrensaft—Agar: KH_2PO_4 1,0 g, Glukose 5,0 g, Pepton 5,0 g, Agar 20,0 g, Möhrenaftbrühe 1000 ml.

Der Einfluss der *verschiedenen Kohlenstoffquellen* auf die Antibiotikumproduktion wurde auf folgendem Nährboden untersucht: K_2HPO_4 0,1%, $MgSO_4$ 0,05%, NaCl 0,05%, $FeSO_4$ 0,001%, $CaCl_2$ 0,03%, $(NH_4)_2HPO_4$ 0,1%, C-Quelle 0,5%, p_H 6,8—7,0.

Experimenteller Teil

Isolierung des Actinomyces-Stammes M 17

Aus einem Mistbeetboden aus Budapest wurden 8 Aktinomyzeten- und 11 Bakterienstämme isoliert. Die gegenseitige antibiotische Wirksamkeit dieser Stämme wurde auf einem Bouillon-Pepton-Glukose-Agar mit Hilfe der vitalen Testmethode festgestellt [1]. Die Ergebnisse sind in Tabelle I zusammengestellt. Die Hemmwirkung der in der ersten waagerechten Reihe ange-

führten — verschieden numerierten — Stämme auf die verschiedenen Stämme der ersten senkrechten Reihe können in den senkrechten Reihen abgelesen werden.

Tabelle I

Die gegenseitige antibiotische Wirksamkeit von 8 Aktinomyzeten- und 11 Bakterienstämmen auf Bouillon-Pepton-Glukose-Agar

	Strahlenpilze								Bakterien										
	M3	M4	M5	M8	M13	M15	M17	M19	M25	M27	M30	M31	M32	M33	M34	M36	M41	M43	M45
M3	0	0	0	0	0	0	7	0	0	g	2	3	0	g	0	g	0	5	5
M4	0	0	0	0	5	0	6	0	0	4	g	g	0	g	0	4	0	0	3
M5	0	0	0	3	4	0	16	0	0	0	5	3	0	2	0	4	0	g	3
M8	0	2	4	0	7	0	15	0	0	3	6	5	6	5	0	3	0	0	3
M13	0	4	0	0	0	0	7	0	0	0	5	g	g	5	0	4	0	4	3
M15	2	0	0	0	12	0	20	0	0	2	g	g	0	3	0	5	0	0	g
M17	2	0	0	0	6	0	0	0	0	0	g	3	0	3	0	g	0	0	5
M19	2	0	0	0	2	0	8	0	0	0	g	3	0	4	0	4	0	g	g
M25	3	0	0	4	7	0	0	0	0	0	0	3	0	0	0	g	6	0	5
M27	0	0	0	0	7	0	0	0	0	0	4	3	0	4	0	4	2	0	8
M30	0	0	0	0	3	g	0	0	0	0	0	0	0	4	0	3	0	0	5
M31	2	0	0	0	9	0	0	0	0	0	2	0	0	5	0	10	0	0	3
M32	6	0	0	0	10	3	0	0	0	g	4	3	0	3	0	g	0	0	3
M33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M34	8	0	0	0	3	3	7	0	0	0	0	4	0	3	0	g	0	0	0
M36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M41	0	0	0	0	3	0	0	0	0	0	0	2	0	4	0	4	0	3	5
M43	0	0	0	0	15	0	0	0	0	0	0	3	0	0	0	3	g	0	g
M45	3	g	0	0	6	0	0	0	0	0	0	0	0	0	0	g	0	0	0

Die in den Quadraten stehenden Zahlen bezeichnen den Halbmesser der Hemmzonen in mm.
g = schwache Hemmung von ungefähr 1 mm

Aus den Angaben der Tabelle ist ersichtlich, dass die Aktivität der Strahlenpilzstämme (M 3—19) gegenüber den Aktinomyzeten und Bakterien (M 25—45) ihres eigenen Bodens sehr unterschiedlich ist. Die Aktinomyzete M 5 war nur gegenüber dem Strahlenpilz M 8 wirksam. Der Stamm M 13 war den Bakterien und den Aktinomyzeten gegenüber gleicherweise aktiv. Wie zu sehen ist, sind das Hemm- und Empfindlichkeitsspektrum jedes einzelnen Stammes

jeweils anders, und bei der Auswahl der Kulturen waren es diese zweifachen Spektrumunterschiede und nicht so sehr die systematischen Merkmale, die ins Gewicht fielen.

Der mit M 17 bezeichnete Actinomyces-Stamm hemmte beträchtlich sämtliche Strahlenpilzstämme seines eigenen Bodens in ihrer Entwicklung. Am aktivsten erwies er sich dem Stamm M 15 gegenüber, der laut unserer Bestimmung [2] der Art *A. griseus Krainsky 1914* am nächsten steht. Ausserdem hemmte er die Entwicklung des grampositiven, nicht säurebeständigen, am Anfang aus sich verzweigenden Stäbchen und dann aus zu Kokken zerfallenden Kulturen bestehenden Stammes M 34, der sich laut unserer Bestimmung als die Art *Mycobacterium mucosum Krassilnikow 1941* erwies. Die Tatsache, dass andere Testorganismen den antibiotischen Substanzen des Stammes M 17

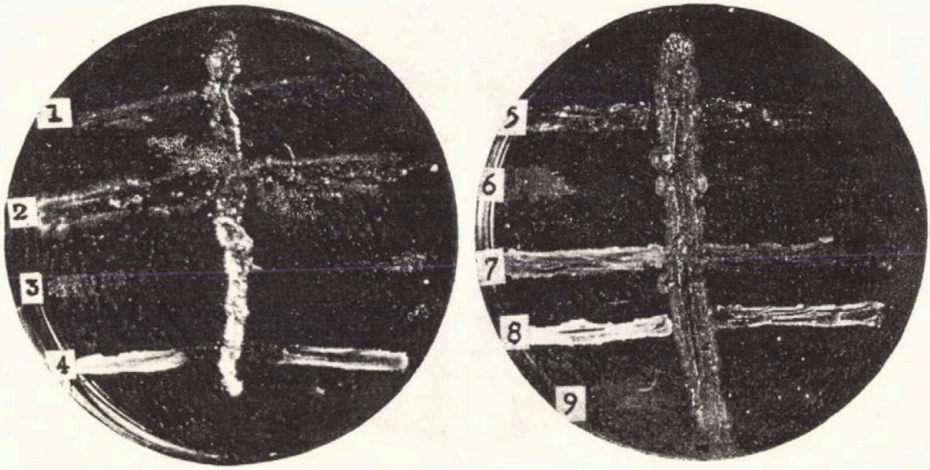

Abb. 1. Empfindlichkeit verschiedener Versuchsmikroorganismen gegenüber dem antibiotischen Stoff von Actinomyces M 17
(Bezeichnungen der Teststämme. 1: Escherichia coli. 2: Bacillus subtilis. 3: Actinomyces griseus M 15. 4: Saccharomyces cerevisiae. 5: Sarcina lutea. 6: Mycobacterium mucosum M 34. 7: Staphylococcus aureus. 8: St. albus. 9: Serratia marcescens

gegenüber empfindlich sind, geht aus Abb. 1 hervor, wo dieser Stamm auf Bouillon-Pepton-Glukose-Agar mit Hilfe des WAKSMANschen Kreuztestes untersucht wurde.

Aus der Abbildung geht deutlich hervor, dass sich die Hemmwirkung des Stammes M 17 bloss in die Richtung von *Actinomyces* und *Mycobacterium* erstreckt. Die schwache Entwicklung von *S. cerevisiae* in der Nähe von Actinomyces M 17 war, wie dies die späteren Untersuchungen bestätigten, nicht auf antibiotische Einflüsse zurückzuführen.

Auf Grund dieser Ergebnisse schien es von Interesse, den Stamm Actinomyces M 17 einer eingehenderen Untersuchung zu unterwerfen.

Die Morphologie von Actinomyces M 17

Der Stamm Actinomyces M 17 bildet auf Glukose-Asparagin-Agar (Glukose 10 g, Asparagin 0,5 g, K_2HPO_4 0,5 g, Agar 15 g, dest. Wasser 1000 ml, pH 6,2—6,4) bereits am 3.—4. Tage seiner Entwicklung an den Luftmyzelien spirale Sporophoren aus. Die zwei bis vier Windungen beschreibenden Spiralen (Abb. 3) sind an den Luftmyzelien in grösseren oder kleineren Abständen voneinander rankenartig angeordnet. Die Dicke der Fäden des Luftmyzels beträgt 1 bis 1,2 μ, die Sporen sind kugelförmig und haben einen Durchmesser von etwa 1 μ.

Auf JENSENschem *Kasein-Glukose-Nährmedium* weisen die Kolonien eine grauweisse Farbe auf, in ihrer Mitte befindet sich eine kräftige, steck-

Abb. 2. Kolonien von Actinomyces M 17 auf JENSENschem Kasein-Glukose-Nährboden

nadelkopfgrosse Erhebung, gegen die Ränder zu lässt sich ein feines Ringmuster (1—2 mm) erkennen, das in einen sich spärlich erweiternden mehlartigen Überzug übergeht (Abb. 2). Auf KRASSILNIKOWschem *synthetischem Agar Nr. III* [3] ergeben sich ockergelbe Kolonien, die stellenweise mit weissem Luftmyzel bedeckt sind. Auf KRASSILNIKOWschem *synthetischen Agar Nr. IV* [3] erhält man sich gut entwickelnde, zusammenhängende, mit weissem Luftmyzel bedeckte Kolonien. Actinomyces M 17 gibt auf keinem dieser synthetischen Medien Farbstoff an den umgebenden Nährboden ab. Auf *Glyzerin-Natriumnitrat-Agar* [4] von der Zusammensetzung K_2HPO_4 0,1%, $MgSO_4$ 0,05%, KCl 0,05%, $FeSO_4$ 0,001%, $NaNO_3$ 0,2%, Glyzerin 3,0%, Agar 1,5% entstehen gut entwickelte Kolonien, die reich mit weissem Luftmyzel bedeckt sind, von unten gesehen eine gelbe Farbe aufweisen und keinen Farbstoff an die Umgebung

abgeben. Auf dem bereits erwähnten *Glukose-Asparagin-Agar* entwickeln sich mit dichtem, weissem, später grau werdendem Luftmyzel bedeckte Kolonien, eine Verfärbung des Nährmediums konnte auch hier nicht beobachtet werden. Auf dem gleichfalls eingangs angegebenen *Möhrensaft-Agar* sind die Kolonien braun, am Anfang stellenweise mit weissem Luftmyzel bedeckt, das später grau wird, die Kolonien sind an ihrer Unterseite flechtenartig, narbig und geben braunen Farbstoff an den Nährboden ab. Ebenfalls bräunlichgelbe Kolonien mit weissen Luftmyzelflecken sind an *Kartoffelblöcken* zu sehen. Aus der Kolonie

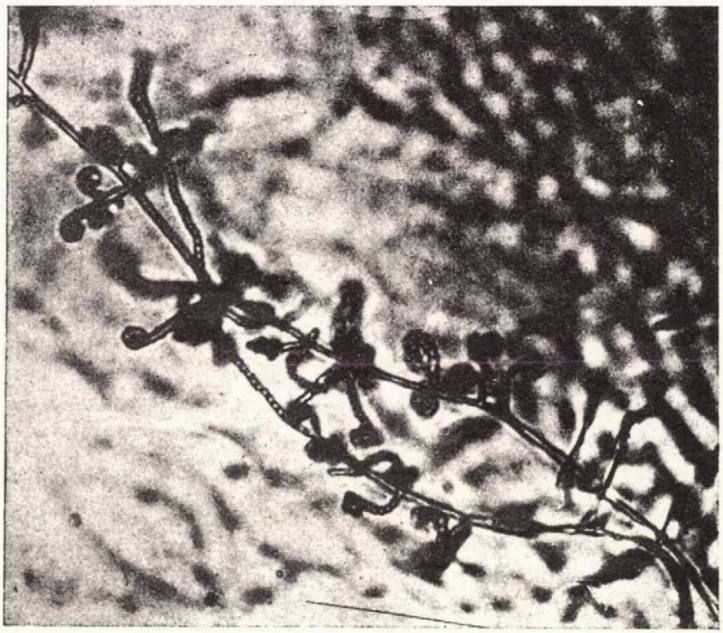

Abb. 3. Sporophoren am Luftmyzel von Actinomyces M 17

diffundiert schwarzer Farbstoff in die Kartoffel. Auf WAKSMANschem *Nährboden* [2] erhält man gut gedeihende Kolonien mit weissem Luftmyzel, das später grau wird. Der Nährboden verfärbt sich, indem er eine braune Farbe annimmt. Auf *Bouillon-Pepton-Glukose-Nährboden* [1] ergeben sich gut wachsende, graue, mit narbenartigen Falten durchzogene Kolonien, hier bildet sich kein Luftmyzel. Auch hier erscheint der braune Farbstoff im Nährmedium. Eine Bildung von braunem Farbstoff war auch in *Milch* wahrzunehmen. In flüssigen Schüttelkulturen (WAKSMANsches Nährmedium und Bouillon-Pepton-Glukose-Medium, im weiteren *Bouillon X* genannt) sind die Kolonien kugelförmig, laichartig.

Die Physiologie von Actinomyces M 17

Wie bekannt, hindert die Variabilität der Actinomyces-Arten und -Stämme in zahlreichen Fällen die Bestrebungen, eine genaue morphologische und physiologische Beschreibung von ihnen zu geben. Infolge dieser Variabilität dürfte auch die Intensität ihrer Stoffwechselprozesse und deren Richtung beträchtlichen Veränderungen ausgesetzt sein. Laut unserer Untersuchungen zeichnen sich die von uns isolierten und kultivierten Stämme von Actinomyces M 17 durch einer *ausgesprochener Stabilität ihrer Erbeigenschaften* aus. Diese Tatsache besitzt für die weiteren Untersuchungen eine sehr grosse Bedeutung, denn so war es möglich, die verhältnismässig stabile Natur der wichtigsten Artmerkmale (Antibiotikumproduktion, Sporenbildungsfähigkeit, physiologische Eigenschaften) während der bereits mehr als ein Jahr dauernden Untersuchungen festzustellen.

Verwertung der C-Quellen : Bei Anwesenheit einer anorganischen N-Quelle z. B. $(NH_4)_2HPO_4$ zersetzt der Stamm Actinomyces M 17 die *Zellulose*. Er gedeiht gut auf *Glykogen*. Er hydrolysiert die *Stärke*. Er verwertet ausgezeichnet *Raffinose*, *Maltose* und *Laktose*. Er invertiert *Saccharose*. Als eine der besten Kohlenstoffquellen erwies sich die *Zellobiose*. Eine ausgezeichnete Entwicklung konnte auf den Aldohexosen *(d-Glukose, d-Mannose, d-Galaktose)* festgestellt werden, doch erwies sich auch die *d-Fruktose* als ähnlich gute Kohlenstoffquelle. Von der in die Gruppe der Aldopentosen gehörenden *l-Arabinose* und *d-Xylose* erwies sich die erstere gut und die letzteren bereits in vermindertem Ausmass verwertbar. Vom untersuchten *Mannit* und dem diesem stereoisomeren *Sorbit* war ebenfalls der erstere die bessere C-Quelle. Auf *Inosit* wurde ein zufriedenstellendes Wachstum beobachtet. Analog war auch das Ergebnis bei *Glyzerin*. Unter den Salzen der untersuchten organischen Säuren erwies sich das *K-Zitrat* als gute C-Quelle. Interessant ist es, das K-Zitrat hier gleich mit dem *K-Oxalat* zu vergleichen, auf dem die Myzelbildung bereits kaum vor sich ging. Diese Beobachtung stimmt mit den Feststellungen von WAKSMAN [5] über die Verwertungsfähigkeit dieser Kohlenstoffquellen für Strahlenpilze überein. Bei Berücksichtigung der Angaben von STAPP [6] ist allerdings Actinomyces M 17 unter die potenteren Aktinomyzeten zu reihen. Auf *Na-Formiat* ist Actinomyces M 17 (in Schüttelkulturen) gut wahrzunehmen, obgleich nur eine sehr schwache Entwicklung festgestellt wurde.

Verwertung der N-Quellen : in Anwesenheit von Ammoniumphosphat gewährleisteten Natriumnitrat, Ammoniumnitrat, Kalziumnitrat, Ammoniumsulfat als mineralische N-Quellen ein gleichermassen gutes Wachstum. Die kräftigste Entwicklung des Myzeliums konnte in Gegenwart von Fleischextrakt, Pepton, Rübenextrakt usw. festgestellt werden. Wenn man als gemeinsame C- und N-Quelle *Albumine*, *Na-Nukleat* oder *Karbamid* verwendete, war ein schwaches oder gar kein Wachstum zu beobachten. Wurde Glukose als

C-Quelle mit Asparagin als C- und N-Quelle kombiniert, so wurde eine mittelmässige Myzelbildung erzielt, während bei Kombination von Glukose mit Glykokoll ein gesteigertes Wachstum festzustellen war. Wurden Aminosäuren allein als C- und N-Quellen benutzt, so wurde bei *Arginin* eine mittelmässige, bei *Glykokoll*, *Alanin*, *Leuzin*, *Methionin* und *Histidin* eine schwache oder keine Entwicklung gefunden.

Der Stamm Actinomyces M 17 wächst in *Gelatine* — bei Bildung von braunem Farbstoff — sackartig hinein und verflüssigt dieses erst nach einer längeren Zeit (5—6 Wochen). Auf LÖFFLER-Serum erhält man sich gut entwickelnde Kolonien mit faltiger Oberfläche, die wenig braunen Farbstoff bilden.

Züchtungseigenschaften : Ein günstiges Wachstum von Actinomyces M 17 wurde bei pH-Werten zwischen 6 und 7, bei einer Temperatur von 25 bis 30° C auf verschiedenen organischen Medien und synthetischen Nährböden festgestellt, wie z. B. auf verschiedenen sterilisierten, bereicherten, humusarmen Lehm- und Sandböden, an den im Autoklav behandelten Blättern verschiedener Pflanzen (Eiche, Nussbaum, Schilfrohr usw.) an Weizenstroh, an Kuhfäkalien usw. Leider entwickelte sich Actinomyces M 17 auf nichtsterilisierten Medien nicht in verfolgbarer Weise. Diese Erscheinung lässt sich nur durch die mikrobiellen Konkurrenzverhältnisse erklären. Die Züchtungsverhältnisse des Stammes können — auch unter Berücksichtigung seiner Antibiotikumproduktion — sowohl auf Oberflächenkulturen, in Schüttelkulturen als auch in durchlüfteten Fermentorgefässen (von 10 l) unter Rührung günstig gelöst werden. Er ist äusserst empfindlich gegen bakterielle Verunreinigungen, wobei diese Gefahr desto grösser ist, als sein antibiotisches Stoffwechselprodukt sowohl gegen gramnegative wie auch gegen grampositive Bakterien in gleicher Weise wirkungslos ist. Laut unserer Untersuchungen liegt der optimale p_H-Wert bei Beginn der Fermentation zwischen 6,5 und 7,0. Danach ist eine allmähliche Erhöhung des p_H-Wertes zu beobachten, der auf mehreren von uns untersuchten flüssigen Nährmedien (CZAPEK, Bouillon X, »corn-steep« usw.) zwischen dem 8. und 14. Tage 8,0 bzw. höchstens 9,0 erreicht. Die Menge der sich in der Kulturlösung bildenden Myzelien wird nicht nur durch die C- und N-Quellen und die einzelnen anorganischen Salze stark beeinflusst, sondern auch durch den Ausgangswert der Wasserstoffionenkonzentration. In einer Versuchsreihe auf CZAPEK-Nährboden bei 25°C wurde in Schüttelkulturen bei Anwendung von je 200 ml Kulturlösung folgendes festgestellt : wenn der Ausgangswert des p_H 6,85 betrug, wog die Myzeltrockensubstanz am 14. Tage 1,7118 g bei einem p_H-Wert von 7,83. Wenn dagegen der Ausgangswert des p_H 6,18 ausmachte, war die gesamte Trockensubstanz im erwähnten Zeitpunkt 0,8058 g bei einem p_H-Wert von 7,64. Schliesslich war nach einem anfänglichen p_H-Wert von 5,26 das Gewicht der Myzeltrockensubstanz am 14. Tage 0,4844 g, wobei sich der p_H-Wert auf 7,19 erhöht hatte. Trotz dieser beträchtlichen Unterschiede des Myzelgewichtes war die antibiotische Aktivität in allen drei Fällen gleich gross.

Die Bedeutung des antibiotischen Metabolits im Stoffwechsel von Actinomyces M 17

Das Wirkungsspektrum. Eine sehr grosse Zahl von Untersuchungsergebnissen spricht dafür, dass das antibiotische Wirkungsspektrum und die antibiotische Aktivität wesentlich von den quantitativen und qualitativen Verhältnissen des benutzten Nährmediums abhängen können. Es ist ausserdem auch die Diskussion bekannt, die über die Bewertung der natürlichen Rolle der Antibiotika entstanden ist und im Laufe derer zahlreiche Forscher die Meinung vertreten, dass die Antibiotika entweder infolge der ungewohnten Umstände des künstlichen Nährbodens entstandene abnormale Stoffwechselprodukte sind, oder jedoch normale, aber intermediäre Stoffwechselprodukte, die infolge der Wirkung der besonderen Verhältnisse der Nährböden produziert werden. In keinem Falle sind aber die Antibiotika ursprünglich antibakterielle Stoffe.

Bei der hier untersuchten Art wurde die Klarstellung dieser Frage vor allem mit der Feststellung des antibiotischen Wirkungsspektrums auf dem komplexen Bouillon X (pH:6,8) Nährboden in Angriff genommen. Mit der zum Nachweis der Empfindlichkeit herangezogenen Mikrobe wurden Platten gegossen und die Stückchen des Myzels von Actinomyces M 17 mit Hilfe einer Öse auf die erstarrte Oberfläche der Platten aufgebracht. Die Ergebnisse werden in Tabelle II gezeigt, wo 0 das Ausbleiben der Hemmung, ein + eine Hemmzone von 5 mm Halbmesser, zwei + einen von 10 mm Halbmesser, 3 + einen von 15 mm Halbmesser und 4 + einen von 20 mm Halbmesser bedeuten.

Wie ersichtlich sind die antibiotischen Stoffwechselprodukte von Actinomyces M 17 den herangezogenen grampositiven und gramnegativen Bakterien und den Kokken gegenüber vollständig wirkungslos. Ähnlicherweise konnte auch in keinem einzigen Falle eine Hemmung gegenüber Hefen beobachtet werden. Diese Untersuchungen wurden auch auf mehrere solche Actinomyces-Stämme ausgedehnt, die aus den von FEHÉR aus den verschiedensten Erdteilen beschafften Bodenproben isoliert wurden. Der Herkunftsort der Bodenproben und die Zahl des Stammes sind jeweils in Klammern angeführt. Sämtliche untersuchten Actinomyces-Stämme und determinierten Arten waren gegenüber den antibiotischen Stoffwechselprodukten von Actinomyces M 17 empfindlich. Ebenfalls konnte bei den mit den Strahlenpilzen verwandten Proactinomyces- und Mycobacterium-Arten eine Empfindlichkeit nachgewiesen werden. Obwohl hinsichtlich der Pilze kein einheitliches Ergebnis erzielt wurde, so darf man immerhin auf Grund des Gesamtbildes die Feststellung machen, dass der in den Nährboden diffundierende Wirkstoff in erster Linie auf die Strahlenpilze eine Hemmwirkung ausübt.

Wirkungsspektrum und Kohlenstoffquelle. Um festzustellen, wie sich das antibiotische Wirkungsspektrum bei Verwendung der verschiedenen Kohlen-

Tabelle II

Antibiotisches Wirkungsspektrum von Actinomyces M 17 auf Bouillon-X-Agar

Testorganismus	Halbmesser der Hemmzone
1. Agrobacterium radiobacter	0
Bacillus subtilis	0
Escherichia coli	0
Serratia marcescens	0
Sarcina lutea	0
Staphylococcus albus	0
Staphylococcus aureus	0
2. Actinomyces aureofaciens	++
Actinomyces fradii	++
Actinomyces griseus M 15	+++++
Actinomyces globisporus streptomycini	++
Actinomyces rimosus	++
Actinomyces sp. (Boghari 2)	++
Actinomyces sp. (Halland-Väderö)	++
Actinomyces sp. (In-Ecker 4)	+++++
Actinomyces sp. (Jawa 1)	+++++
Actinomyces sp. (Jawa 2)	+++
Actinomyces sp. (Krakatau 1)	++
Actinomyces sp. (Krakatau 3)	++
Actinomyces sp. (Krakatau 4)	+++++
Actinomyces sp. (Pemberton 1)	+++++
Actinomyces sp. (Pemberton 4)	+++++
Actinomyces sp. (Sba 1)	++
Actinomyces sp. (Sba 3)	++
Actinomyces sp. (Tanezrouft 4)	+++
Actinomyces sp. (Tanezrouft 5)	++
Actinomyces sp. (Tanezrouft 8)	+++
Actinomyces sp. (Tanezrouft 9)	++
Mycobacterium sp.	++
Mycobacterium mucosum M 34	++
Proactinomyces roseus	++
Proactinomyces sp.	+++
3. Candida sp.	0
Hansenula anomala	0
Saccharomyces cerevisiae	0
Saccharomyces chevalieri	0
Saccharomyces italicus	0
Torulopsis utilis	0
Zygosaccharomyces Marxianus	0
4. Absidia ramosa	0
Aspergillus sp.	++
Aspergillus ruber	+
Aspergillus versicolor	0
Botrytis cinerea	++
Fusarium sp.	+
Penicillium citreo-viride	+
Penicillium granulatum	+
Penicillium waksmani	0
Penicillium chrysogenum	++

stoffquellen gestaltet, wurde folgender Versuch ausgeführt. Mit verschiedenen C-Quellen versehenes flüssiges Nährmedium (Zusammensetzung siehe im Abschnitt »Material und Methoden«) wurde in Mengen von je 8 ml in Reagensgläser eingefüllt, mit den Sporen von Actinomyces M 17 beimpft und die Reagenzgläser zur besseren Durchlüftung auf eine zu ihrer Aufnahme geeignete drehbare Scheibe gelegt. Nach 6 Tagen wurde der Inhalt der Röhrchen zentrifugiert und die Aktivität der Kulturflüssigkeit mit dem Lochtest gegen die Mikroben *E. coli*, *B. subtilis*, *St. aureus*, *Sarc. lutea*, *Mycobact. mucosum M 34*, *Actinom. griseus M 15*, *Penicillium chrysogenum* und *Sacch. cerevisiae* untersucht. Die Verlässlichkeit dieser Methode wurde zu wiederholten Malen überprüft, wobei festgestellt wurde, dass man mit keiner Wirkstoffproduktion der in der Kulturlösung eventuell zurückbleibenden Myzelien während der 16- bis 25stündigen Untersuchung zu rechnen hat. Die mit Hilfe von Asbestfiltern (z. B. Filtrox Steril S 2408) ausgeführten Filtrierversuche zeitigten kein positives Ergebnis, da der Wirkstoff durch diese nicht hindurchdrang. In der Zwischenzeit wurde die Feststellung gemacht, dass das fragliche antibiotische Stoffwechselprodukt stark thermostabil ist und dass man die Kulturflüssigkeit bei 120° C sterilisieren kann. Die Abnahme der antibiotischen Aktivität der Kulturflüssigkeiten nach Wärmebehandlung liess sich — bei Messung mit der Agardiffusionsmethode — nur bei übrigens sehr niedrigen Hemmwerten (2—6 mm) deutlich feststellen.

Auf Grund dieser Ergebnisse wurden die Untersuchungen derart modifiziert, dass die Empfindlichkeit sämtlicher obenerwähnter Arten gegenüber dem nicht mit Wärme behandelten Zentrifugaten geprüft wurde, ferner die Empfindlichkeit von *B. subtilis* und *Act. griseus M 15* auch noch gegenüber der bei 120° C sterilisierten Fermentflüssigkeit. Die erhaltenen Resultate sind in Tabelle III zusammengestellt.

Da in der Aktivität der mit den zweierlei Methoden behandelten Kulturflüssigkeiten kein wesentlicher Unterschied festgestellt wurde, sind die mit den zwei Methoden erhaltenen Ergebnisse auch nicht gesondert in der Tabelle angeführt. Aus den Angaben der Tabelle III geht hervor, dass in keinem einzigen Falle eine Hemmwirkung gegen *E. coli* noch gegen *B. subtilis*, *St. aureus*, *Sarc. lutea*, noch gegen *Sacch. cerevisiae* nachgewiesen werden konnte. Demgegenüber war die Richtung der antibiotischen Aktivität bei sämtlichen Kohlenstoffquellen die gleiche. Am empfindlichsten erwies sich *Act. griseus* M 15, während die Hemmung von *Mycobact. mucosum M 34* und *Penicillium chrysogenum* je nach der Produktion des Wirkstoffes eintrat oder ausblieb. Die unter den obenerwähnten Versuchsverhältnissen durchgeführten fortlaufenden Untersuchungen zeitigten das Ergebnis, dass auf jenen Kohlenstoffquellen, auf denen die Myzeliumbildung gering war (Zellulose, Sorbit, K-Zitrat), die am sechsten Tage gemessene niedrige antibiotische Aktivität langsam weiter zunahm.

Tabelle III

Das Wirkungsspektrum von Actinomyces M 17-Kulturen, die auf verschiedenen C-haltigen Nährböden gezüchtet wurden

C-Quelle	E. coli	B. subtilis	St. aureus	Sarcina lutea	Mycobact. mucosum	Act. griseus	Penic. chrysogenum	Sacch. cerevisiae
Zellulose	—	—	—	—	—	6	—	—
Glykogen	—	—	—	—	7	16	3	—
Amylum	—	—	—	—	10	16	8	—
Raffinose	—	—	—	—	8	16	10	—
Laktose	—	—	—	—	9	17	7	—
Saccharose	—	—	—	—	9	15	10	—
Maltose	—	—	—	—	6	14	4	—
Zellobiose	—	—	—	—	9	17	7	—
Glukose	—	—	—	—	9	17	7	—
Galaktose	—	—	—	—	2	10	—	—
Mannose	—	—	—	—	7	15	9	—
Fruktose	—	—	—	—	9	16	6	—
Mannit	—	—	—	—	9	15	9	—
Sorbit	—	—	—	—	—	9	—	—
Arabinose	—	—	—	—	10	14	6	—
Xylose	—	—	—	—	—	8	—	—
Glyzerin	—	—	—	—	8	15	7	—
K-Zitrat	—	—	—	—	3	11	—	—
K-Oxalat	—	—	—	—	—	4	—	—
Na-Formiat	—	—	—	—	—	5	—	—
Karbamid	—	—	—	—	—	—	—	—

Antibiotikumproduktion: Da die Absicht bestand, die Umstände der Antibiotikumproduktion unter den Verhältnissen des Bodens zu erforschen, musste man sich vor allem zumindest in groben Zügen einen Begriff vom Ablauf der Produktion machen. Bei unseren Untersuchungen mit flüssigen Schüttelkulturen wurde schon früher festgestellt, dass die alternden Kulturen einen zweiten antibiotischen Wirkstoff oder Wirkstoffkomplex an das umgebende Medium abgeben, allerdings nicht auf jedem Nährboden. So wurde das Erscheinen dieser zweiten Fraktion auf synthetischen Medien nicht beobachtet, während sie auf Bouillon X und auf WAKSMANschem Nährboden in den Schüttelkulturen nach 6 oder 7 Tagen auftrat.

In Tabelle IV sind die auf den synthetischen KRASSILNIKOWschen Nährböden Nr. III und Nr. IV und auf dem WAKSMANschen Nährboden nach neuntägigem Schütteln festgestellten Aktivitätsergebnisse zusammengefasst.

Tabelle IV

Das Wirkungsspektrum von Actinomyces-M-17-Kulturen, die auf verschiedenen Nährböden gezüchtet wurden

Nährboden	E. coli	B. subtilis	St. aureus	Sarcina lutea	Mycobact. mucosum	Act. griseus	Penic. chrysogenum	Saech. cerevisiae
Weizenstroh	—	1	1	—	7	15	2	—
Nussbaumblatt	—	2	1	—	—	10	—	—
Schilfblatt	—	—	—	—	—	10	—	—
Eichenblatt	—	—	—	—	—	6	—	—
WAKSMAN-Nährboden ..	—	3	2	—	7	16	8	—
KRASSILNIKOW-III-Nährboden	—	—	—	—	7	16	7	—
KRASSILNIKOW-IV-Nährboden	—	—	—	—	4	12	4	—

Es ist ersichtlich, dass sich das Wirkungsspektrum auf dem WAKSMANschen Nährboden in der Richtung gegen *B. subtilis* und *St. aureus* ausbreitet. Ausserdem sind in Tabelle IV auch die Resultate der mit Blättern verschiedener Pflanzen ausgeführten Versuche enthalten. In 1000-ml-Erlenmeyerkolben wurden je 125 ml des von FEHÉR [7] zur Untersuchung des Zelluloseabbaus empfohlenen $CaCO_3$-haltigen Nährmediums eingemessen. In jeden Kolben wurden je 5 g trockenes, kurzgeschnittenes Weizenstroh oder Nussbaum-, Schilf- bzw. Eichenblätter eingelegt. Danach wurde sterilisiert, beimpft und ihre antibiotische Aktivität nach 9 Tagen mit den Lochtest-Methoden untersucht. Wie aus der Tabelle hervorgeht, erwies sich sowohl das Weizenstroh als auch das Nussbaumblatt als ein geeignetes Medium zur Produktion der zweiten antibiotischen Fraktion. Es ist jedoch zu bemerken, dass sowohl die Versuche mit dem Stroh als auch mit den Blättern in unbeweglichen Kulturen erfolgten.

Im Laufe der weiteren Untersuchungen erwies es sich als notwendig, die zwei Fraktionen voneinander zu trennen. Die erste, deren Wirkung sich gegen Actinomyces, Mycobacterium und die Pilzarten richtete, ging nicht durch den Asbestfilter Filtrox Steril S 2408 hindurch und erwies sich als thermostabil. Die zweite, gegen *B. subtilis* und *St. aureus* wirksame Fraktion ging durch diesen Filter hindurch und war thermolabil. Zur besseren Unterscheidung wurde die erste Fraktion, deren Wirkung sich in erster Linie gegen Actinomyces richtete, »Anti-Actinomyces-Faktor« und die zweite »A. M. 17« genannt. Die chemische Isolierung dieser Fraktionen wird parallel zu unseren Arbeiten von I. OROSZLÁN durchgeführt.

Danach wurde eine Versuchsreihe angesetzt, um die Kurve der »Anti-Actinomyces-Faktor«-Erzeugung festzustellen. Die diesbezüglichen Ergebnisse sind in Abb. 4 veranschaulicht.

Insgesamt wurde mit sieben Nährmedien gearbeitet, nämlich : 1. Bouillon X, 2. WAKSMANsches Nähremedium, 3. CZAPEKsches Nährmedium, 4. »Corn-steep«-Glukose-Nährmedium, 5. Möhrenextrakt-Glukose-Pepton-Nährmedium, 6. Tyrosin-Glukose-Ammoniumsulfat-Nährmedium, 7. Glukose-Ammoniumsulfat-Nährmedium. Die Fermentation erfolgte in Schüttelkulturen von je 200 ml bei 28° C bei einem anfänglichen pH-Wert von 7,0. Die Zunahme der antibiotischen Aktivität der Kulturflüssigkeiten gegenüber *Act. griseus M 15* wurde mit den Lochtestmethoden ermittelt und in mm des Halbmessers der Hemmzonen ausgedrückt (Abb. 4).

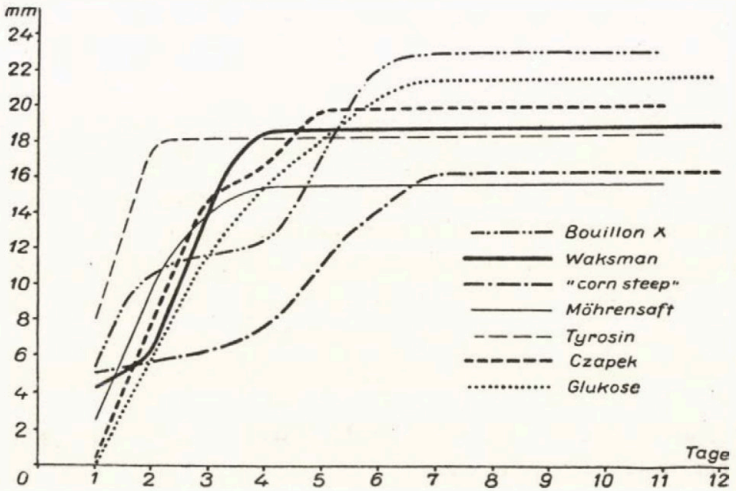

Abb. 4. Die Produktion des Anti-Actinomyces-Faktors als Funktion der Zeit auf verschiedenen Nährböden

Wie ersichtlich setzt die Produktion insbesondere auf dem Tyrosin enthaltenden Nährboden ziemlich schnell ein, erreicht zwischen dem 4. und 7. Tage ihr Maximum, um von da an auf einem unveränderten Wert zu bleiben. Bei den Nährmedien CZAPEK, »Corn-steep« und Bouillon X wurde vor Erreichen des Maximums eine Einsenkung der Kurve beobachtet, deren Ursache nicht bekannt ist. Bei den Kontrollversuchen konnte diese Erscheinung nicht so deutlich wahrgenommen werden. Die Angaben der Abb. 4 weisen darauf hin, dass der Aktivitätswert, bei dem die Produktion bereits unverändert bleibt, bei den einzelnen Nährmedien sehr verschieden sein kann. Die grösste Aktivität wurde bei der Bouillon X festgestellt, die schwächste bei dem Möhrenextrakt.

Antibiotikumproduktion im Boden. Es bleibt somit noch die Frage zu untersuchen, ob die Verhältnisse des Bodens eine Möglichkeit zur Antibiotikumproduktion gewähren. Naturgemäss hängt dies auch von den physikalisch-chemischen Verhältnissen des Bodens ab. So liessen sich zahlreiche mit diesem

Ziel angesetzte erfolglose Versuche dadurch erklären, dass zu den Kulturen nicht der geeignetste Bodentyp gewählt wurde. In Tabelle V wird über jene Versuche berichtet, im Laufe derer auf zwei Bodentypen versucht wurde, die Produktion der antibiotischen Stoffe zu bestätigen. Der erste Boden stammte aus einem Eichenpark und enthielt 2,9% Humus, während der zweite Boden Komposterde aus einem Mistbeet mit 5,0% Humusgehalt war. (Die Humusbestimmung erfolgte nach Extraktion mit NaOH durch oxydimetrisches Titrieren.)

Im weiteren wurde folgenderweise vorgegangen: je 40 g Boden wurden in 200-ml-Erlenmeyerkolben eingemessen, bis 50% der maximalen Wasserkapazität befeuchtet und in einzelnen Versuchsreihen noch 2% Weizenstroh bzw. Stärke, Glukose oder Sojamehl hinzugefügt. Dann wurden diese Proben bei 120° C sterilisiert und danach mit Sporensuspension beimpft. Die Inkubation der Kulturen erfolgte im Thermostaten bei 27° C während 32 Tage, wobei eine Entwicklung von Actinomyces M 17 auf den sterilisierten Böden mit dem blossen Auge festgestellt werden konnte. Während der einen Monat langen Versuchszeit wurde regelmässig für den Ersatz der verdunsteten Wassermenge gesorgt. Am 32. Versuchstag wurden in jeden Kolben je 50 ml steriles dest. Wasser eingemessen, die Kolben mit einem Gummistopfen verschlossen und 30 Min. auf der Schüttelmaschine geschüttelt. Nach dem Setzen des Niederschlages wurde die über dem Boden stehende Flüssigkeit abpipettiert, zentrifugiert und ihre Aktivität gegenüber *E. coli*, *B. subtilis* und *Act. griseus M 15* mit dem Lochtest geprüft. Die Angaben der Tabelle V zeigen die Hemmwirkung der einzelnen Bodenextrakte an.

Tabelle V

Die antibiotische Wirksamkeit der wässerigen Extrakte von verschieden behandelten Bodenproben

Bodentyp	Behandlung der Bodenprobe	Hemmwirkung des wässerigen Bodenextraktes in mm gegenüber		
		Escherichia coli	Actinomyces griseus M 15	Bacillus subtilis
Lehmboden	Ohne Zugabe	—	7	—
	+ 2% Weizenstroh ..	—	8	—
	+ 2% Stärke	—	9	—
	Kontrolle	—	—	—
Mistbeetboden	Ohne Zugabe	—	—	—
	+ 2% Glukose	—	10	—
	+ 2% Sojamehl	—	5	—
	Kontrolle	—	—	—

Diese Untersuchungen wurden auch mit Bodenextrakten durchgeführt, die bei 120° C sterilisiert worden waren. Das Spektrum dieser Proben war völlig mit dem der unsterilisierten identisch, nur war die Wirkung etwas geringer. Der sterile Lehmboden ohne Zugabe von Weizenstroh usw. löste eine Hemmzone von 7 mm Halbmesser auf die Entwicklung von *Act. griseus M 15* aus, wogegen er gegenüber E. coli und B. subtilis unwirksam war. Eine noch höhere Produktion des antibiotischen Stoffes konnte auf den mit Weizenstroh bzw. Stärke ergänzten Böden beobachtet werden. Im sterilen Mistbeetboden ermöglichte nur die Ergänzung mit Glukose und mit Sojamehl eine Produktion des Wirkstoffes. Bei den sterilisierten Böden waren die aus den unbeimpften Kontrollen hergestellten Extrakte wirkungslos.

Biotische Inaktivierung : Da es nicht gelang, im natürlichen, mit Hitze nicht sterilisierten Boden eine Produktion des Antibiotikums nachzuweisen, wurde versucht, die Ursache für dieses Ausbleiben zu erforschen. Es schien möglich, dass sich der Actinomyces-Stamm M 17 in diesen Böden, wenn auch schwächer, sich doch zu entwickeln begann, wobei aber der produzierte Wirkstoff von der ursprünglichen Mikroflora abgebaut oder unwirksam gemacht wurde. Es musste also die Stabilität des angenommenen »Anti-Actinomyces-Faktors« in Gegenwart der verschiedenen Mikroorganismen festgestellt werden. Das hierbei von uns angewandte Verfahren war im wesentlichen die zum gleichen Zweck ausgearbeitete Methode von WALLHÄUSER [8]. Es wurde also folgendermassen vorgegangen : 200 ml flüssiges WAKSMANches Nährmedium wurde mit einer Sporensuspension von Actinomyces M 17 beimpft und in einem Erlenmeyerkolben 6 Tage hindurch geschüttelt. Dann wurde die aktive Kulturflüssigkeit zentrifugiert, mit frisch bereitetem WAKSMANschem Nährmedium auf das Zweifache verdünnt und bei 120° C (Moment-Autoklavierung) sterilisiert. Danach wurden je 20 ml der Flüssigkeit in 100-ml-Erlenmeyerkolben eingefüllt und mit den in Tabelle VI angeführten Mikroorganismen beimpft.

Das Beimpfen erfolgte am 15. Juni. Am 20. konnte ein gutes Wachstum sämtlicher Mikroben festgestellt werden. Nach 7 Tagen wurde der Inhalt eines Teiles der Kolben zentrifugiert, sterilisiert und seine Aktivität gegen *Act. griseus M 15* und *B. subtilis* mit dem Lochtest untersucht. Ein anderer Teil des Versuchsmaterials wurde nach 2 Wochen auf seine antibiotische Aktivität geprüft. Beim ersten Test (22. VI.) wurde im Vergleich zu der einen Halbmesser von 14 mm aufweisenden Hemmzone der Kontrollen nur in einem einzigen Fall, nämlich bei *B. subtilis*, eine unbedeutende Inaktivierung beobachtet. In allen anderen Fällen verringerte sich — trotz des guten Wachstums der eingeimpften Organismen — die gegen Actinomyces gerichtete hohe Wirkung der Kulturflüssigkeit nicht. Dies bedeutet, dass dieselben Strahlenpilze, die im Anfangsstadium ihres Wachstums eine hohe Empfindlichkeit gegen den statisch wirksamen »Anti-Actinomyces-Faktor« zeigen, später auch in Gegenwart dieses Hemmstoffes zu wachsen imstande sind, ohne diesen deshalb seiner Wirksam-

Tabelle VI

Die Wirksamkeit verschiedener Mikroorganismen auf den »Anti-Actinomyces-Faktor«

Zeitpunkt der Untersuchung	22. VI.		29. VI.	
Versuchs-mikroorganismus	Halbmesser der Hemmzone in mm gegenüber			
	Act. griseus	B. subtilis	Act. griseus	B. subtilis
Kontrolle	14	—	12	—
Act. sp. M 3	14	5	12	3
Act. sp. M 5	14	—	12	—
Act. sp. M 15	14	4	12	3
Act. sp. M 13	14	7	12	6
Myc. muc. M 34	14	—	12	—
B. subtilis	13	—	8	—
E. coli	14	—	12	—
St. aureus.....	14	—	12	—
Sarcina lutea	14	—	12	—

keit zu berauben. Die Actinomyces-Stämme M 3, M 15 und M 13 erzeugen auch selber — wie aus Tabelle VI ersichtlich — thermostabile, aber bereits gegen B. subtilis wirksame antibiotische Stoffe. Anlässlich der am Ende der zweiten Woche durchgeführten Untersuchungen wurde festgestellt, dass die antibiotische Aktivität der Kulturflüssigkeiten zusammen mit der der Kontrollen etwas gesunken war. Von einer biotischen Inaktivierung kann man aber noch immer nur bei B. subtilis sprechen, doch geht auch dieser Prozess sehr langsam vor sich.

Besprechung der Ergebnisse

Im Zusammenhang mit den obigen Ergebnissen sind zwei Probleme eingehender zu besprechen. Das erste ist die gegenseitige Hemmung der Actinomyces-Arten. Laut SZABÓ [9] muss in der Natur ein gesteigerter Antagonismus zwischen den Strahlenpilzen bestehen, da diese die hinsichtlich ihrer ökologischen Ansprüche einander am nächsten stehenden Organismen sind und sowohl ihre Populationsdynamik als auch ihre Rolle in der Mineralisation von denjenigen der Bakterien verschieden ist. Im Sinne dieser Deutung darf man die strahlenpilzfeindliche Wirkung von Act. M 17 unter gewissen Umständen — unter Berücksichtigung der massgeblichen Rolle dieses Hemmstoffes im Stoffwechsel — als einen wirksamen Faktor im Antagonismus dieses Stammes anderen Aktinomyzeten gegenüber betrachten.

Das andere Problem ist die Frage der Antibiotikumproduktion unter den Verhältnissen im Boden. In unserem Falle war es im nichtsterilen Boden nicht möglich, eine Antibiotikumproduktion nachzuweisen, obwohl sich der betreffende Wirkstoff als recht resistent gegen die biotische Inaktivierung erwies. Hierbei ist auch in Betracht zu ziehen, dass der Boden ein ausserordentlich heterogenes System darstellt, in dem sich die Bodenmikroben in den einen oder den anderen der kleinen Mikrobenherde hineinziehen und dort ihre Tätigkeit ausüben. Der Fall, wo ein Mikroorganismus in irgendeinem Boden eine derartige Alleinherrschaft erreicht, dass sich seine Stoffwechselprodukte in einem leicht nachweisbaren Ausmass anhäufen, muss als eine Seltenheit angesehen werden. Um jedoch die Stoffwechselprodukte der Mikrobenherde, deren Durchmesser in einer Grössenordnung von Bruchteilen eines Millimeters liegen, nachweisen zu können, sind unsere Methoden noch zu anfänglich.

* * *

Hier sei Akademiker D. Fehér für die Unterstützung unserer Arbeit der beste Dank ausgesprochen

Zusammenfassung

1. Im Laufe unserer Untersuchungen wurde ein als »Actinomyces M 17« bezeichneter Strahlenpilzstamm ausgezüchtet, der ein Antibiotikum mit einem besonderen Wirkungsspektrum produziert. Dieser Wirkstoff (»Anti-Actinomyces-Faktor«) übt die stärkste Aktivität gerade gegenüber den Strahlenpilzen aus, während er sich gegen eine ganze Reihe von gram. positiven und gramnegativen Bakterien als wirkungslos erwies.

2. Dieser thermostabile, eine statische Wirkung zeitigende und der biotischen Inaktivierung gegenüber überaus resistente Hemmstoff bildet das essentielle Stoffwechselprodukt von Actinomyces M 17 und wird auf den verschiedensten Kohlenstoffquellen und auf allen Nährmedien — den natürlichen, humusarmen Lehmboden mit inbegriffen — abgeheben, auf den sich dieser Strahlenpilz überhaupt zu entwickeln vermag.

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ВЗАИМНЫЙ АНТАГОНИЗМ ЛУЧИСТЫХ ГРИБКОВ

I. Исследование штамма Actinomyces M 17

И. САБО и М. МАРТОН

Резюме

1. В ходе опытов авторы выделили штамм лучистых грибков **Actinomyces M 17**, который производит обладающий особенным спектром действия антибиотик. Это действующее вещество (**«Anti-Actinomyces-factor»**) оказывается наиболее активным в отношении лучистых грибков, но неэффективен по отношению к целому ряду грам-положительных и грам-отрицательных бактерий.

2. Это термостабильное, тормозящее статическое действие вещество, весьма резистентное к биотическому инактивированию, является эссенциальным продуктом обмена веществ штамма **Actinomyces M 17** и образуется в различных источниках углерода и на всякой такой питательной среде — включая и бедную гумусом естественную почву — где вообще могут развиваться эти лучистые грибки.

LEPTOSPIROSENHERDE IN OSTUNGARN

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Nach der genauen Identifikation der in verschiedenen Gebieten Transdanubiens auftretenden Leptospirenepidemien durch ALFÖLDY, FÜZI und Mitarbeiter [1—4] sowie nach ihrer systematischen epidemiologischen Aufarbeitung durch diese Forscher durfte angenommen werden, dass die Leptospirose auch auf das Gebiet jenseits der Theiss in Form kleinerer oder grösserer Epidemien übergreifen wird. Die ersten diesbezüglichen Untersuchungen im Gebiet jenseits der Theiss gehen gleichfalls auf ALFÖLDY zurück, indem er nämlich im Jahre 1943 bei Untersuchung des Serums eines typhusverdächtigen Theissfischers (aus dem Komitate Szolnok) eine positive Leptospirenagglutination nachwies. Im gleichen Jahre stellte er auch Versuche mit 16 Mäusen aus den Reisfeldern der Pussta Hortobágy an, wobei sich mit dem Serum eines dieser Mäuse eine positive Leptospirenagglutination ergab.

Die im Jahre 1951 auf dem Gebiete Hortobágy auftretenden schweren Leptospirenepidemien wurden von KELEN [6] entdeckt und ausführlich beschrieben. Er berichtete über 5 Epidemien, die in den Staatsgütern Elep, Szásztelek, Ohát und Geszt aufgetreten waren. Die Krankheit griff hauptsächlich junge, noch saugende Kälber des ungarischen Fleckviehs an, wobei 50 bis 60% des Bestandes von ihr befallen wurde. Der Verlust betrug 10 bis 20%. Unter den Kälbern trat die Krankheit epidemisch zur Zeit des Weideganges auf, in der Winterperiode (November—März) konnte sie nur sporadisch festgestellt werden. Vom epidemiologischen Gesichtspunkt war es von Interesse, dass es auf den Weiden der Kälber zahlreiche abflusslose, oberflächliche Gewässer und Tümpel gab, aus denen die Kälber ein durch infizierten Urin verunreinigtes Trinkwasser erhielten. Gleichzeitig lebten auf diesen Gebieten auch sehr viele Nagetiere (Mäuse, Hamster und vornehmlich Ratten), u. zw. sowohl innerhalb als auch ausserhalb der Ställe.

Das erste epidemische Auftreten der menschlichen Leptospirose im Gebiet jenseits der Theiss wurde von FAZEKAS und BÍRÓ [7] im August 1952 im Dorfe Bakonszeg (Komitat Hajdú—Bihar) entdeckt. Die Mehrzahl der Erkrankten waren junge Knaben, etwa 40 an der Zahl, vereinzelt wurden auch 1 oder 2 Mädchen bzw. 1 oder 2 Erwachsene von der Krankheit befallen. Die Erkrankten

hatten in dem damals seichten Fluss gebadet, wobei etwa 500 m oberhalb der Badestelle die Schweineherde der Gemeinde zu baden pflegte. Nur solche Personen wurden von der Krankheit befallen, die unterhalb des Badelplatzes der Schweineherde im Fluss gebadet hatten. Mit dem Serum von 5 Kranken ergab sich ein positives Agglutinationsergebnis, aus dem Blute von 3 Kranken konnten Leptospiren, aus dem von 2 Kranken *L. pomona* und aus dem eines Kranken *L. grippothyposa* gezüchtet werden.

Um festzustellen, in welchem Ausmass die Leptospirose in dem Gebiet jenseits der Theiss vorkommt, wurde an die Ärzte der Umgebung ein Rundschreiben gerichtet. In diesem wurden die Symptome der Leptospirose beschrieben und die Versandvorschriften für die zu den Laboratoriumsprüfungen notwendigen Untersuchungsmaterialien angegeben. Ferner wurden die Merkmale der Leptospirenepidemien und die anzuwendende Therapie kurz geschildert. Gleichzeitig wurden die Ärzte ersucht, unserem Institute eine Mitteilung zu machen, falls eine solche Erkrankung in ihrem Bezirk vorgekommen war oder vorkommt. Das Ergebnis dieses Aufrufes war, dass von mehreren Ärzten entsprechende Mitteilungen und Untersuchungsmaterial einliefen.

Aus den eingegangenen Seren wurden Verdünnungen von 1 : 100 hergestellt und mit dieser Grundlösung Agglutinationsversuche mit 9 verschiedenen Leptospirenstämmen angestellt. Im Falle, wo Agglutination oder Lysis auftrat, wurden aus den Seren Verdünnungsreihen hergestellt und deren Agglutinationstiter bestimmt.

Parallel mit der Bestimmung der Agglutination im Dunkelfeld wurde unter der Leitung von Prof. JENEY der Agglutinationstiter auch in Kollargolpräparaten abgelesen. Zur Bereitung des kollargolhaltigen Ausstriches wurde 10%iges Kollargol mit einer Öse auf einen entfetteten Objektträger gebracht, daneben eine Öse Flüssigkeit aus dem Agglutinat aufgetragen, die beiden Präparate auf dem Objektträger vermischt und mit dem Deckglas dünn ausgestrichen. Nach Trocknen erfolgte die Ablesung mit einem Immersionsobjektiv. Bei Lysis wurden jeweils in einem Dunkelfeld Leptospirenteile und sporadisch 1 oder 2 Leptospiren beobachtet. Bei Agglutination waren dagegen die charakteristischen Gruppen und Geflechte der zusammengeballten Leptospiren im Ausstrich zu sehen. In negativen Fällen kamen die Leptospiren vollständig gesondert vor.

Im Laufe der durchgeführten Untersuchungen erwies sich die Auswertung der Leptospirenagglutination mit dem Kollargolausstrich als ein rascheres und bequemeres Verfahren als die Auswertung im Dunkelfeld. Zur Züchtung wurde ein Nährboden nach KORTHOF benutzt, wobei die beimpften Nährböden alle 5 Tage im Dunkelfeld und im Kollargolpräparat geprüft wurden.

Als Ergebnis des obenerwähnten Rundschreibens lief von den Kreisärzten Nachricht über die folgenden Erkrankungen und Epidemien ein :

In *Nagylêta* erkrankten vom 15. Juni bis 4. Juli 1953 etwa 30 Kinder im Alter von 8 bis 14 Jahren. Unter den Beschwerden der Kranken kamen in jedem einzelnen Fall ein mit Schüttelfrost, mit nachfolgendem plötzlichen hohen Fieber und Brechreiz einhergehender Krankheitsbeginn, Kopf- und Gliederschmerzen, Genickstarre, Schläfrigkeit und in einzelnen Fällen Sprechstörungen vor. Das hohe, um 40° C schwankende Fieber hielt 4 bis 5 Tage an, wonach eine 1—2tägige fieberfreie Periode und dann wieder eine 1 bis 2 Tage dauernde Fieberperiode folgte. Der spontane Ablauf der Krankheit konnte nur in wenigen Fällen beobachtet werden, da die Ärzte in den meisten Fällen durch Verabreichung von Penicillin eingriffen. Auf Wirkung dieses Antibiotikums hörte die Krankheit nach 1 bis 2 Tagen auf. Die einzelnen Gemeindeärzte dachten zuerst an eine Sommerinfluenza, an Grippe. In der Anamnese der kranken Kinder kam aber in jedem einzelnen Falle das regelmässige Baden in dem am Rande der Ortschaft vorbeifliessenden und zum Baden der Rinder und Schweine benutzten Kanal vor, was dann neben den obigen Symptomen den Verdacht auf Leptospirose lenkte. Bei einer von unserem Institut an Ort und Stelle durchgeführten Erhebung wurden 10 kranke Kinder auch physikalisch untersucht. In einem Teil der Fälle wurde Genickstarre beobachtet, sonst konnte nur Fieber von etwa 40° C konstatiert werden. Für die Laboratoriumsdiagnose wurde Blut von den weniger als eine Woche kranken Kindern in Nährboden nach KORTHOF zu Züchtungszwecken überimpft und von den gleichen Kranken auch zitrathaltiges Blut genommen, in dessen zentrifugiertem Niederschlag nach Leptospiren geforscht wurde. Die Agglutinationsprobe mit dem Serum von 5 Kranken zeitigte ein positives Ergebnis : im Serum des Patienten K. J. wurde bei einer Verdünnung von 1 : 6400 *L. pomona*, in dem des Patienten B. G. bei einer Verdünnung von 1 : 6400 *L. pomona* und bei einer Verdünnung von 1 : 800 *L. kelen* (von KELEN im Jahre 1952 anlässlich einer Rinderepidemie im Gebiete Hortobágy aus einem Kalb gezüchtet), in dem des Patienten F. J. bei einer Verdünnung von 1 : 6400 *L. pomona* und bei einer solchen von 1 : 1600 *L. kelen*, in dem des Patienten B. D. bei einer Verdünnung von 1 : 1600 *L. kelen* und schliesslich in dem des Patienten M. J. bei einer Verdünnung von 1 : 200 *L. pomona* festgestellt. Nach einem Monat wurde eine zweite Erhebung in dieser Ortschaft durchgeführt, bei der aber nur von 3 Kranken Blut zu einer wiederholten Untersuchung genommen werden konnte. Von den 3 Seren zeigte nur das von K. J. eine positive Agglutination mit *L. pomona* bei einer Verdünnung von 1 : 12 800. Dieses Serum war schon bei der vorigen Agglutination in einer Titer von 1 : 6400 positiv.

Aus dem Blute von drei Kranken gelang es, Leptospiren zu züchten. In zwei Fällen konnten in dem Blute, das am zweiten bzw. dritten Tage der Krankheit genommen wurde, am 15. Züchtungstag Leptospiren nachgewiesen werden, während beim dritten Kranken das Blut am 6. Tage der Krankheit — am 1. Tage der zweiten Fieberperiode — genommen und die Anwesenheit von Leptospiren

nach 21tägiger Inkubation beobachtet wurde. Alle drei Stämme erwiesen sich bei der Identifikation als *L. pomona.*

Zwischen dem 10. und 30. Juli 1954 kamen etwa 40 Erkrankungen in der Ortschaft *Derecske* vor. Es wurden hauptsächlich Kinder von 10 bis 14 Jahren von der Krankheit befallen, doch konnte sie auch an einigen Erwachsenen beobachtet werden. In der Anamnese der Kranken kam auch hier der plötzliche Krankheitsbeginn mit hohem Fieber und starken Kopf- und Wadenschmerzen vor. Die Kranken heilten rasch nach Gabe von 300 000 E Penicillin. Die Gemeindeärzte beobachteten bei einem Grossteil der Kranken Genickstarre. Anlässlich einer von unserem Institut durchgeführten Erhebung an Ort und Stelle wurden 17 Kinder und Erwachsene untersucht. Bei den Kranken wurde hohes Fieber (39,6—40,2° C) und Genickstarre festgestellt. In der Anamese eines Teiles der Kranken kam Baden in dem neben dem Dorfe fliessenden Kanal vor, wobei in einer Entfernung von 200 bzw. 500 m oberhalb der Badestelle die Schweine- und Rinderherde des Dorfes gebadet wurden. Der andere Teil der Kranken hatte in den am Rande der Ortschaft befindlichen Tümpel gebadet, durch die die Rinder- und Schweineherden hindurchgetrieben zu werden pflegten und wo die Schweineherde gebadet wurde.

In 4 von den untersuchten 17 Fällen ergab sich im pathognostischen Titer eine Agglutination. Das Serum des 9jährigen Patienten D. T. gab in einer Verdünnung von 1 : 12 800, das des 12jährigen Patienten Á. L. in einer solchen von 1 : 6400, das des 11jährigen Patienten K. S. in einer solchen von 1 : 1600 und das des 14jährigen Patienten Gy. I. in einer solchen von 1 : 400 eine positive Agglutination mit *L. pomona.* Zusammen mit dem Blute dieser Kranken wurde auch eine Agglutinationsprobe mit dem Serum von zwei gesunden Personen vorgenommen, die zusammen mit den Erkrankten gebadet hatten, doch war hier das Ergebnis negativ. Hämokulturen wurden nur mit dem Blut von zwei Kranken angesetzt, da nur bei diesen die Krankheit jünger als eine Woche war. In beiden Fällen war das Ergebnis negativ.

Sowohl bei den Kranken aus der Ortschaft Nagyléta wie bei denen aus Derecske wurden mit dem Niederschlag des Zentrifugats von zitrathaltigem Blut Untersuchungen im Dunkelfeld und in Kollargolpräparaten durchgeführt. Es gelang in keinem einzigen Falle, Leptospiren nachzuweisen.

Aus der Ortschaft *Aporliget* (Komitat Szabolcs) wurden drei Fälle gemeldet. F. J. wurde 8 bis 10 Tage, nachdem er zusammen mit zwei Gefährten in einem Teich gebadet hatte, von der Krankheit befallen. Nach zwei Tagen erkrankten auch seine Gefährten. F. J. stand an der II. Medizinischen Klinik in Debrecen in Behandlung. Die Untersuchung seines Serums mit *L. pomona* ergab bei einer Verdünnung von 1 : 400 eine positive Agglutination, während sich die Hämokultur als negativ erwies.

In der Klinik für Nervenkrankheiten in Debrecen wurden von SAMU Untersuchungen über das Ausmass durchgeführt, in dem die Leptospirose unter

den Fällen von »serösen Gehirnhautentzündungen« vorkommt. Im Jahre 1953 standen 18 an seröser Meningitis erkrankte Personen an dieser Klinik in Behandlung. Das Blut von 7 dieser Patienten wurde in unserem Institut hinsichtlich der Leptospirenagglutination untersucht, wobei in 4 Fällen eine positive Agglutination beobachtet wurde. Alle vier Seren agglutinierten *L. pomona*. Bei der Prüfung der Erkrankungsumstände der auf die Nervenklinik aufgenommenen Patienten wurde in einem Falle ein gehäuftes Vorkommen der Krankheit festgestellt, nämlich bei 6 Schweinehirten eines staatlichen Schweinezuchtbetriebes. Diese betreuten dieselbe Herde und erkrankten im gleichen Zeitpunkt. Sie badeten die Schweine und standen dabei selber bis zu den Knien im Wasser, in dem sie die Schweine badeten. Von ihnen erkrankte nur eine einzige Person schwer. Drei Tage nach dem Erscheinen der klinischen Symptome wurde diesem Schweinehirten Blut entnommen: in einer Verdünnung von 1 : 800 agglutinierte sein Serum mit *L. pomona*, während 10 Tage später mit demselben Stamm eine Agglutination bei einem Titer von 1 : 12 800 festgestellt wurde. Dieser Patient hatte keine Penicillinbehandlung erhalten.

Im Staatsgut in *Berettyóújfalu* trat anfangs Juli 1953 unter den Rindern eine Leptospirenepidemie auf. Zuerst zeigte sich bei drei Kühen Hämoglobinurie, später erkrankten mehrere (etwa 35) Kälber. Von den Kälbern gingen etwa 10 Stück ein oder mussten notgeschlachtet werden. Nach Penicillin- und Ultraseptylbehandlung heilten die Tiere gut. Aus der Niere der eingegangenen Tiere wurden im Veterinärhygienischen Institut in Debrecen mit der von SZÉKI [8] modifizierten, beschleunigten LEVADITIschen Silberimprägnation die Leptospiren in grosser Zahl nachgewiesen. Im Verein mit dem Veterinärhygienischen Institut in Debrecen führte unser Institut zur Gewährleistung einer retrospektiven Diagnose ungefähr 1 Monat nach dem Erlöschen der Epidemie mit dem Blut von 120 kranken und nichtkranken Tieren Leptospirenagglutinationen durch. Die Seren ergaben in 20 Fällen eine positive Agglutination, u. zw. in 6 Fällen mit *L. pomona*, in 7 mit *L. australis*, in 2 mit *L. grippotyphosa*, in 1 Fall mit *L. bataviensis*, in 1 Fall mit *L. sejrő* und in 3 Fällen mit *L. barta* (ein vom Epidemiologischen Institut der Veterinärwissenchaftlichen Hochschule erhaltener, aus Ratten gezüchteter Typ).

Es ist zu erwähnen, dass die Epidemie im Sommer 1953 in jenem Teil des Staatsgutes Berettyóújfalu ausbrach, der am Ufer des Flusses Berettyó nahe zur Ortschaft *Bakonyszeg* gelegen ist, wo im Jahre 1952 eine menschliche Leptospirenepidemie aufgetreten war.

Um Angaben über das Ausmass zu erhalten, in dem die Tierpfleger der Infektionsgefahr ausgesetzt sind und in dem sie die Erkrankung überstehen können, wurden mit dem Blut von 15 Tierpflegern und 1 Tierarzt, die sich mit den kranken Tieren befassten, Leptospirenagglutinationen durchgeführt. Das Serum einer Person ergab eine positive Agglutination, u. zw. bei einer Verdünnung von 1 : 1600 mit *L. pomona* und bei einer solchen von 1 : 200 mit *L.*

grippotyphosa. Dieser Kranke wies auch die für Leptospirose charakteristischen leichteren Symptome auf. Das Blut des Tierarztes ergab bei einer Verdünnung von 1 : 100 mit *L. sejrő* und das Blut eines Tierpflegers bei einer Verdünnung von 1 : 100 mit *L. pomona* eine positive Agglutination. Da bei diesen Personen vorher keine auf Leptospirose hinweisenden Symptome vorgekommen waren, ist anzunehmen, dass sich bei den gefährdeten Werktätigen eine gewisse Immunität auszubilden vermag.

Um die Frage des Resevoirs klarzustellen, wurden in Nagyléta Nagetiere eingefangen. Bisher gelang es, insgesamt 195 Mäuse zu sammeln, die alle der Art *Microtus arvalis* angehörten. Von diesen wurde mit dem Blute einer Maus bei einer Verdünnung von 1 : 200 mit *L. pomona* und mit dem Blute einer anderen Maus bei einer Verdünnung von 1 : 100 mit *L. sejrő* eine positive Agglutination erhalten. In der Niere der ersten Maus wies das Veterinärhygienische Institut mit der LEVADITIschen Silberimprägnation eine grosse Zahl von Leptospiren nach und bei diesem Falle zeitigte auch die Untersuchung des Blaseninhaltes im Dunkelfeld ein positives Resultat. Die Züchtungsversuche aus Niere und Blase schlossen dagegen in jedem Fall mit einem negativen Ergebnis.

Anlässlich der Erhebungen in Nagyléta wurde auch der Urin der am Ufer des Kanals weidenden und vermutlich dort auch badenden Tiere (Schweine und Rinder) gesammelt. Aus dem Urin von 114 Rindern und 56 Schweinen wurden je 3 Proben in Nährboden nach KORTHOF geimpft. Die Ergebnisse wurden einen Monat hindurch alle 5 Tage bei Untersuchung im Dunkelfeld und in Tuschpräparaten abgelesen. Alle Züchtungsversuche schlossen mit einem negativen Ergebnis, wahrscheinlich deswegen, weil die Saprophytenbakterien die Leptospiren unterdrückten.

Gleichfalls ein negatives Resultat zeitigten die Züchtungsversuche, die mit dem Blaseninhalt von 162 St. im Schlachthof von Debrecen geschlachteten Schweinen angestellt wurden.

Unterzieht man die Verteilung der Leptospirosenerkrankungen nach dem Alter der betroffenen Personen im Gebiete jenseits der Theiss einer Prüfung, so ergibt sich, dass hauptsächlich jüngere Kinder, solche im Alter von 8 bis 14 Jahren erkrankten und dass unter diesen die Krankheit auch am häufigsten epidemisch auftrat. Unter den Erwachsenen kam die Leptospirose bloss sporadisch vor, nur im Schweinezuchtbetrieb von Tetétlen konnte sie unter Erwachsenen gehäuft (6 Personen) und gleichzeitig festgestellt werden. Vom Gesichtspunkt der Beschäftigung waren es vornehmlich Schulkinder, die erkrankten, u. zw. solche, die in infizierten Gewässern badeten. Unter den Erwachsenen verteilten sich die Kranken wie folgt auf die verschiedenen Beschäftigungszweige : 8 Schweinehirten, 2 Angestellte eines staatlichen Schweinezuchtbetriebes, 1 Erdarbeiter, 1 Bahnarbeiter, 1 Taglöhner und andere landwirtschaftliche Arbeiter.

Die Erkrankungen traten zum grössten Teil saisonbedingt, in der Sommerperiode auf. Im Laufe unserer Untersuchungen kamen sie in den Monaten Juli

und August gehäuft epidemisch vor. Die Leptospirose kann aber auch nichtsaisonbedingt auftreten, weil in jenen landwirtschaftlichen Betrieben, wo sie während der Sommerperiode Erkrankungen unter den Tieren hervorgerufen hat, die geheilten oder die Krankheit überstandenen Tiere die Krankheitserreger noch lange Zeit mit ihrem Urin ausscheiden können. So wurde noch im Oktober ein Krankheitsfall an einem Angestellten eines Schweinezuchtbetriebes beobachtet.

Betrachtet man die territoriale Verteilung der Leptospirosenfälle im Gebiete jenseits der Theiss, so ergibt sich, dass die Leptospirose in ganz Ostungarn vorkommt. Sie ist vor allem an der Berettyó endemisch, ausserdem trat die Krankheit in Schweinzuchtbetrieben (Hortobágy) oder neben wenig Wasser führenden Kanälen sowie in Gegenden mit Sümpfen und Tümpeln (Derecske, Nagyléta) auf. Die territoriale Streuung der untersuchten Herde lässt die Möglichkeit zu, dass auf dem ganzen Gebiete jenseits der Theiss neue Leptospirosenepidemien auftreten, so dass die Leptospirose in ganz Ostungarn endemisch vorkommen kann.

An dieser Stelle sei Herrn Fachtierarzt L. Buza für seine weitgehende Unterstützung unserer Arbeiten der beste Dank ausgesprochen.

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ПРИРОДНЫЕ ОЧАГИ ЛЕПТОСПИРОЗА В ЗАТИСЬЕ

Л. СЕНЕШШ и Ж. БИРО

Резюме

Авторы разработали материал эпидемии лептоспироза, появляющихся в Затисье. Среди заболевших в преобладающем большинстве были 8—14 летние дети, купавшиеся в таких каналах, где обычно купали свиней и крупный рогатый скот. Сыворотки исследованных больных в большинстве случаев агглютинировали со штаммом Leptospira pomona. Возбудителя изолировали из крови трех больных, в том числе в двух случаях положительный результат был получен при культивировании крови, взятой на второй и третий день заболевания и в одном случае взятой на шестой день болезни. Все три штамма оказались L. pomona.

При оценке агглютинации авторы — наряду со считыванием в темном поле зрения — производили считывание также в колларголовом мазке. Этот метод оказался более быстрым, удобным и настолько же точным как микроскопия в темном поле зрения.

С целью выявления источников заражения авторы инкубировали в среде Кортгофа мочу большого числа свиней и рогатого скота. Лептоспир однако выделить не удалось. На территории охваченной эпидемией словили 195 мышей (Microtus arvalis). В гистологическом срезе почек одной мыши были обнаружены лептоспиры. Кровь этой мыши в разведении 1 : 200 агглютинировала штамм L. pomona, кровь другой мыши в разведении 1 : 100 агглютинировала штамм L. sejrő. Культивирование, произведенное из почек и из содержимого мочевого пузыря мышей дало отрицательный результат.

Ввиду территориальной разбросанности исследованных очагов, наличие лептоспироза можно считать эндемическим во всем Затисье.

A SEROLOGICAL METHOD FOR ISOLATION AND QUANTITATIVE ESTIMATION OF TRANSFUSED ERYTHROCYTES

By

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ASHBY [1] was the first to describe a serological method for the demonstration of transfused erythrocytes. The essence of his methods is that from the mixture of donor and recipient blood the recipient's erythrocytes are agglutinated with a high-titre agglutinating serum and the non-agglutinated red cells (*i. e.* those of the donor) are counted. The method has been accepted by numerous workers, with slight modifications [2]. The method developed by WIENER is based on a similar principle and involves the use of high-titre anti-M and anti-N agglutinating rabbit sera [3]. MOLLISON has made use of differences within the Rh blood group system in his method for the demonstration of transfused erythrocytes.

In addition to serological methods more recently attemps have been made to utilise radioactive isotopes for labelling and observing erythrocytes circulating in the blood of the recipient [5]. Radioactive iron is built into the haemoglobin of the donor's erythrocytes to make these detectable by physical methods.

The above described procedures have been extensively used mainly in studies on the viability *in vivo* of transfused red cells and also in studies into the aetiology of certain pathological conditions (anaemias).

In the following we describe a method for the isolation and quantitative estimation of transfused erythrocytes.

The essence of the method may be outlined as follows. The red cells of the donor and of the recipient are separated by means of serial differential filtration and the cell count of filtrates containing the donor's red cells is determined in a Bürker chamber. The filter papers used for differential filtration are impregnated with serum agglutinating the red cells of the recipient.

Materials required

1. Pipettes, 0,1 ml, calibrated 1/1000, and 2 ml, calibrated 1/100.
2. A standardized tuberculin syringe.
3. Glass funnels, ∅ 6 cm.
4. Pleated filter papers, M. N. 613, ∅ 6.5 cm.
5. Anti- A and anti-B agglutinating sera with a titre of 1 : 64, avidity within 20″, inactivated at 56° C for 30 min. (The sera should be stored at 4° C in sterile ampoules.)
6. 3,8 per cent disodium citrate. pH 5,5.

Plotting of calibration curve (for determining filtration loss).

1. *Preparation of standard suspensions.* For the determination of four points of the curve, settled A-group «recipient» and B-group «donor» erythrocytes were washed three times in physiological sodium chloride and mixed so in four different ratios that the concentration of B-group red cells be 1, 3, 5 and 10 per cent, respectively. To four Widal tubes containing 0,6 ml each of A-group settled red cells were added 0,006, 0,018, 0,03 and 0,06 ml of B-group settled cells, respectively, to attain the desired concentrations, and then enough citrate to adjust the volumes to 2,5 ml. In addition, a pure suspension of B-group settled red cells was also prepared and 0,06 ml of this, filled up to exactly 2,5 ml with citrate, was used as control

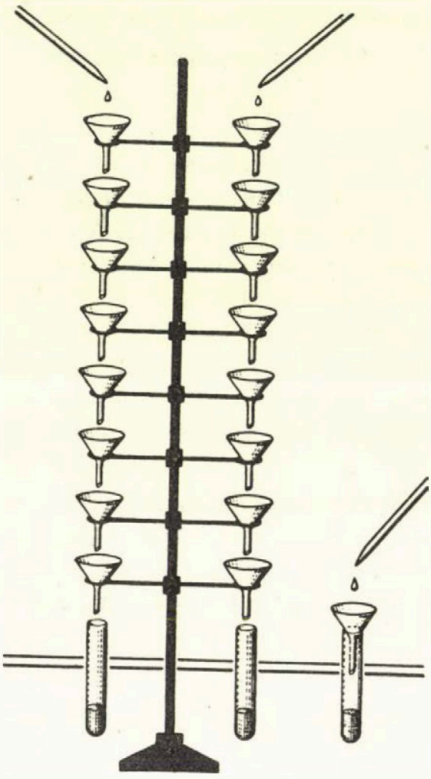

Fig. 1. Schematic presentation of the procedure for filtering blood samples

2. *Filtration.* Except the pure B-group one, the suspensions were filtered, using a differentiating filtering series of 8 funnels for each suspension. The pleated filter papers in the funnels were soaked with anti-A serum, using 1,2 ml for each funnel. For each suspension, 8 funnels were placed one above the other in a vertical row; under the lowest funnel a 20-ml centrifuge tube was placed, serving as receptacle. After homogenisation, each suspension was dripped from a capillary into its own differentiating filtering series. After 10 minutes these were washed with 2 ml of citrate 7 times, at 5-minute intervals and then we waited again until dripping has ceased. Subsequently, the filtrates containing preponderantly donor and only very few recipient red cells were centrifuged at 1000 r. p. m. for 10 minutes and the supernatants were carefully decanted. To the settled erythrocytes so obtained 1 ml citrate was added and the tube thoroughly shaken 8 to 10 times. Then each suspension was passed through a funnel prepared as specified above and after-washed with 1 ml of citrate. The filtrate dripping from the funnel at that time contained exclusively donor red cells, as it could be confirmed by control direct-agglutination and absorption tests. After that, the suspensions were centrifuged again, the supernatants were decanted and the volume in each tube was adjusted to e x a c t l y 2,5 ml. The procedure was carried out at room temperature (+20 to 25° C).

Evaluation

1. *By determining the erythrocyte count.* The red cell count of the sediments adjusted to the above specified volumes (including that of the 10 per cent control) was determined in the small squares of the Bürker chamber, by counting the cells in 200 squares.

2. *Evaluation by plotting of graph.* On the ordinate was indicated the concentration from 0 to 10 per cent, while on the abscissa the red cell count. The

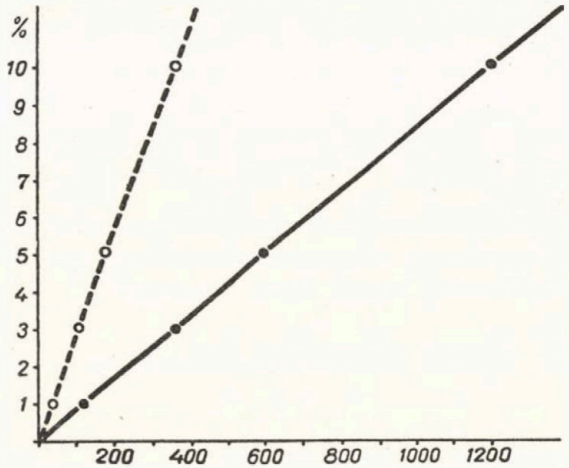

Fig. 2. Curves showing the number of red cells, measured-in and recovered, as determined in a model experiment

mean of ten erythrocyte counts for the B-group control suspension was taken as the 10 per cent value. On the basis of these values was plotted the ideal curve for concentrations from 0 to 10 per cent (without filtration). The percentual filtration yield was calculated from the values of filtered red cell suspensions, as read from the ideal curve. In Fig. 2. are shown the ideal curve and the curve plotted on ground of red cell counts for different filtrates.

Comparison of our procedure with the absorption method

Parallel with the percentual evaluation based on counting, the sediment of isolated donor red cells was examined also by the known absorption and agglutination inhibition methods, in order to facilitate comparison of the two procedures.

The absorptive capacity and agglutination inhibition titre of red cell sediments obtained by differentiating filtration were compared to those of suspensions of known percentual concentration.

The results were in agreement with those of the counting method. As the absorption method is known to be a less precise procedure, in further work the counting method was employed for the purpose of evaluation.

Estimation of red cell count from circulating blood after transfusion

1. *Preparation and filtration of suspensions.* Group 0 blood of known volume was transfused into patients belonging to blood groups A, B or AB. Before transfusion the blood to be transfused was thoroughly homogenized and a sample of it was transferred into a Widal tube. (Preserved blood was used). After 1 hour and then 24 hours following transfusion two 0,6 ml samples of blood were taken from the patient, using a tuberculin syringe. The two samples taken simultaneously (*a*, *b*) were transferred into Widal tubes, each into one tube, containing 1 ml disodium citrate. To sample *a* was added, after thorough homogenization, 0,06 ml of donor blood (control), then the volumes of both suspensions were adjusted to 2,5 ml with citrate. At the same time, a 0,06 ml sample of the blood to be transfused was filled up with citrate to exactly 2,5 ml. (*d*) The percentual concentration of suspension *d* was 10 per cent, as related to the 0,6 ml samples of recipient blood in the other tubes. This examination of the control blood mixture was thought necessary in every experiment, in order to eliminate experimental errors as much as possible. The two suspensions to be filtered (*a* and *b*) were differentiated and a red cell count was made within 1 hour following transfusion. In later tests of the same transfusion fresh preserved blood homologous with the donor blood was used as control, with due regard to differences in red cell count.

2. *Evaluation* was based on the following considerations. From the red cell count of suspension *a* was subtracted that of suspension *b*. The difference gave the postfiltration residual cell count (*c*) of the 0,06 ml donor blood added to suspension *a*. On the basis of the numerical value of suspension *d*, the red cell count of the unfiltered 10 per cent suspension (*d*) was determined. Taking the value of *d* as 100 per cent, the quantity remaining after filtration (expressed in per cents) (*e*) was calculated by the formula

$$\frac{c \cdot 100}{d} = e.$$

On the basis of this percentual value the pre-filtration cell count (i. e. that without loss due to filtration) (*f*) of the transfused blood (*b*) was calculated,

$$\frac{b \cdot 100}{e} = f .$$

In the knowledge of this value, the percentual proportion of the donor blood circulating in the blood of the recipient was obtained by the formula

$$\frac{f \cdot 10}{d} = g,$$

where 10 represents in per cents the concentration of suspension *d*.

Knowing the volume of the blood transfused (*k*), the blood volume of the recipient can be calculated by the formula

$$\frac{k \cdot 100}{g}.$$

If we know the haematocrit value of the pateint's blood, plasma volume can be calculated. In subsequent determinations the volume of transfused blood still present in the recipient was then calculated on the basis of the known value of the recipient's blood volume.

The results of determinations in circulating blood are summarised in Tables I and II.

Table I

Quantitative estimation of volume of transfused and recipient blood

Initials of recipient	Body weight of recipient Kg	Volume of blood transfused ml	Estimated blood volume of recipient 1 hour after transfusion, in litres	Estimated volume of transfused blood after 24 hours, in ml
G. J.	65	310	6,3	295
B. P.	56,50	250	5,2	240
V. J.	68,20	225	6,8	235
Mrs. Sz. J.	38,50	220	3,6	245
Mrs. G. J.	50	245	4,2	245
Mrs. J. J.	56,25	185	4,8	195
B. Gy.	73,75	245	6,4	230
Mrs. G. Gy.	42,50	210	4,5	215

It can be read from Table I that estimations made after 1 hour and 24 hours gave essentially identical values. In order to demonstrate the quantitative reliability of the method, in 3 cases the volume of blood to be transfused was divided into two almost equal parts, of which one was injected 1 hour after transfusion of the other. The determinations were carried out on both occasions. (Table II). On the basis of the data in Table II, the standard error of the method was estimated to be ± 5 per cent.

Table II

Estimation of volume of transfused blood and of patients' blood in parallel experiments

Initials of patient	Body weight of patient, in Kg	Volume of transfused blood, in ml			Blood volume of recipient, in litres	
		1h	2h	Total	1h	2h
K. L.	66,60	115	135	240	6	6,25
A. J.	52,20	100	136	236	5,4	5
V. A.....	59,30	100	90	190	5,8	5,4

Discussion

In the foregoing a serological method has been described for the isolation and quantitative estimation of transfused erythrocytes circulating in the blood of the recipient. The method lends itself also for simultaneous determination of the blood volume of the recipient.

The advantages of our method over the serological methods in use today can be summarized as follows.

Unlike any method based on the differential agglutination principle described by ASHBY, our method makes it possible to isolate transfused red cells. The isolated filtrate may then be subjected to various biological and haematological examinations and thus important information may be obtained as to the aetiology of certain pathological conditions. Moreover, it can be used in tests for controlling the quality of different blood preservatives. The method eliminates the error due to enclosure into agglutinated recipient red cell aggregates of an undeterminable number of donor erythrocytes, which makes the ASHBY method somewhat inaccurate. Also, we can leave the number of non-agglutinated red cells unconsidered when evaluating the results.

As with our method the use of low-titre sera is advantageous, no such high-titre sera are required as for either the ASHBY or the WIENER method. The low-titre serum causes a gradual agglutination of the recipient's red cells and thus the settling agglutinate carries with it a smaller number of recipient erythrocytes.

The use of the method for other purposes and the results of tests to be carried out in the near future will constitute the subject matter of another paper.

Summary

1. A method has been described for the isolation of transfused erythrocytes.

2. The method appears to be suitable for the estimation of the volume of transfused blood.

3. Isolated donor blood may be used in biological and haematological studies.

4. Parallel with differentiating agglutination, the blood volume of the recipient can be determined.

5. The method does not require the use of high-titre agglutinating sera.

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СЕРОЛОГИЧЕСКИЙ МЕТОД ИЗОЛЯЦИИ И КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ ПЕРЕЛИТЫХ ЭРИТРОЦИТОВ

Я. ПЕРКЕДИ, Т. ТАКС и Э. ХОРВАТ

Резюме

1. Авторы описывали метод изоляции перелитых эритроцитов.
2. Метод оказывается пригодным для определения количества перелитой крови.
3. Изолированная кровь донора является пригодной для биологических и гематологических исследований.
4. Одновременно с дифференцировочной агглютинацией возможно произвести также определение количества крови реципиента.
5. При этом методе не имеется необходимости применения агглютинирующей сыворотки высокого титра.

ÜBER DIE WIRKUNG VON SCHWERMETALLEN AUF ATMUNG UND REDOXPOTENTIAL VON STREPTOMYCES AUREOFACIENS-KULTUREN

Von

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(Eingegangen am 10. Januar 1955)

In einer vorhergehenden Mitteilung hatten wir die Wirkung von Schwermetallen auf die Atmung und das Redoxpotential (RP) von *Streptomyces griseus*-Kulturen untersucht [1]. Hierbei wurde festgestellt, dass die Wirkung der aus den Betriebsfermentoren durch Korrosion in den Nährboden gelangenden Schwermetallionen auf den Stoffwechsel der Kulturen erfolgreicher mittels RP-Messungen zu bestimmen ist als durch Atmungsuntersuchungen. Die Messungsergebnisse lassen auch Schlüsse auf die optimale Zusammensetzung des Materials der Betriebsfermentoren vom Standpunkte der Produktion zu.

In der vorliegenden Arbeit haben wir auf Grund der früheren Erfahrungen unsere Untersuchungen auch auf Kulturen des von DUGGAR [2] beschriebenen *Streptomyces aureofaciens*-Stammes ausgedehnt. Hier sollte hauptsächlich untersucht werden, welchen Einfluss die Veränderungen des RP, verursacht durch die infolge von Korrosion aus den Eisenfermentoren in den Nährboden gelegenden Schwermetalle, auf die Atmung dieses Stammes haben, um die dabei gemachten Erfahrungen bei der Aureomycinproduktion praktisch verwerten zu können.

Versuchsmethoden

Die Untersuchungen wurden an dem uns vom Mikrobiologischen Institut der Budapester Agrarwissenschaftlichen Universität zur Verfügung gestellten *Streptomyces aureofaciens*-Stamme angestellt.

Zur Sporenbereitung wurde der Stamm auf folgendem Nährboden gezüchtet (3):

Agar-Agar	15,0 g
Traubenzucker	10,0 g
Asparagin	1,5 g
K_2HPO_4	1,5 g
Dest. Wasser	100 ml, pH 6,8—7,0

Aus den in Reagenzröhrchen auf Schrägagar vorgezüchteten Stämmen wurden mit steriler physiologischer Kochsalzlösung Sporensuspensionen hergestellt und das so gewonnene Inokulum in flüssige Nährböden überimpft.

Der in destilliertem Wasser zubereitete Nährboden hatte folgende Zusammensetzung (4):

Rohrzucker	3,0%
Ammoniumsulfat	0,3%
Calciumcarbonat	0,5%
Corn steep liquor	0,3%

Erdnussmehl (Arachis hypogaea)* 2,0%
Natrium chlorid 0,3%
Melasse 0,2%
pH : 6,6—6,8

* (die Nüsse wurden bei 60 C° getrocknet, mit Petroläther entölt und dann gemahlen)

Die Kulturen wurden während der Versuchszeit (72 Std.) im Pendelschüttelapparat geschüttelt (Geschwindigkeit : 120 Min., Amplitude : 50 mm). Zu den nach der Warburgschen manometrischen Methode vorgenommen Atmungsversuchen wurden in 500 ml-Erlenmeyerkolben in 125 ml Nährboden Vorkulturen gezüchtet. Zu den Atmungsversuchen wurden aus den so hergestellten Vorkulturen 1 : 15 verdünnte Kulturen bereitet und davon 3 ml in jedes Warburg-Gefäss eingetragen. Die Atmung dieser Kulturen wurde im Warburg-Apparat 2 Std. lang bei 29,7—30°C verfolgt. Der in den Versuchen benützte Warburg-Apparat war mit einer speziellen Rührvorrichtung versehen, welche die in Schüttelkulturen gewöhnlich erreichbare Aerifizierung sicherte. Es sei aber bemerkt, dass der Grad der Aerifizierung auch so noch hinter dem des in Betriebsfermentoren erreichbaren zurückbleibt.

Das RP wurde mit einer glatten, einer Kalomelelektrode gegenübergeschalteten Platinelektrode am Metrohm-Titriskop gemessen (die Ergebnisse sind stets auf Normal-Wasserstoffelektrode berechnet angegeben). Die Versuchsbedingungen wurden entsprechend denen der Atmungsversuche gewählt und deshalb die RP-Messungen in offenen Gefässen vorgenommen und zwecks guter Sauerstoffversorgung die Kulturen mit einem magnetischen Rührer gerührt.

Versuchsergebnisse

Es wurde die Wirkung der Ionen folgender Metalle untersucht : Magnesium, Aluminium, Silikat, Vanadium, Mangan, Eisen, Kobalt, Nickel, Kupfer, Zink, Molybdän, Kadmium, Zinn und Blei. Die Ergebnisse gehen aus folgenden Abbildungen hervor.

Wie ersichtlich, wird die Atmung der Kulturen durch Zugabe von 60 γ/ml Eisen und Mangan gesteigert, während Chrom, Blei, Kadmium und Zinn in der gleichen Konzentration eine wesentliche Herabsetzung bewirken, die beiden letzteren sogar bereits in einer Konzentration von 20 γ/ml. Die übrigen Metalle lassen die Atmung unbeeinflusst, ausgenommen das Kupfer, welches — in der Konzentration von 60 γ/ml zugegeben — ausgefällt wird und eine vollkommene Lähmung der Atmung bewirkt (Abb. 1 und 2).

Im weiteren wurde untersucht, welche Metalle eine beständige Veränderung des Redoxpotentials der Kulturen bewirken und welche Konzentrationen sich zur Entfaltung dieses Effektes als notwendig erweisen. Die an Abb. 3 wiedergegebenen Ergebnisse zeigen, dass das RP durch Kupfer, Blei, Eisen und Kadmium gesteigert und durch Vanadium und Zinn herabgesetzt wird, während die übrigen Metalle ohne Einfluss sind. Das auffallendste Resultat unserer Untersuchungen ist, dass das RP der Streptomyces aureofaciens-Kulturen durch die obigen Metallionen in entgegengesetztem Sinne beeinflusst wird wie das der Streptomyces griseus-Kulturen. Aus den Tabellen geht auch hervor, dass die Kulturen sich dem Eisen und Kupfer gegenüber als Redoxpuffer verhalten [1]. Ferner wurde festgestellt, dass gewisse der aus den Eisenfermentoren herausgelösten Metalle, wie z. B. Kupfer und Zinn, sowohl die Atmung, als auch das

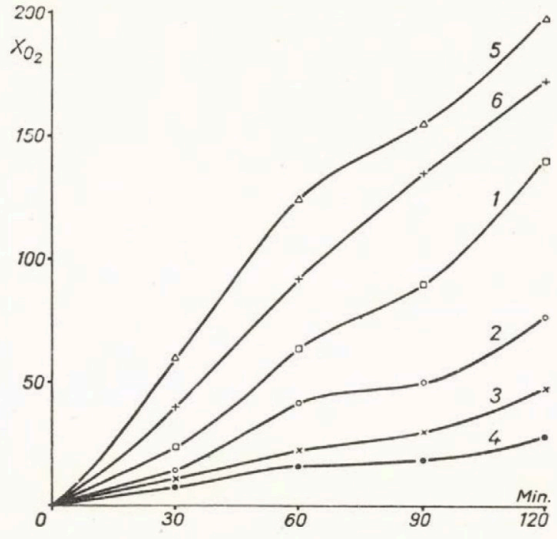

Abb. 1. : Veränderung der Atmung von Streptomyces aureofaciens-Kulturen auf Wirkung von Chrom- und Kadmiumionen

Kurve 1 : Atmung einer Kontrollkultur
« 2 : Veränderung nach Zugabe von 60 Gamma Chrom
« 3 : « « « « 20 « Kadmium
« 4 : « « « « 60 « «
« 5 : « « « « 60 « Eisen
« 6 : « « « « 60 « Mangan

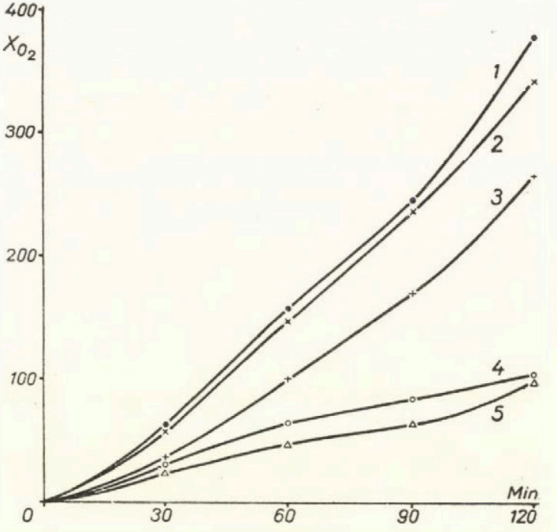

Abb. 2. Veränderung der Atmung von Streptomyces aureofaciens-Kulturen auf Wirkung von Aluminium-, Blei- und Zinnionen

Kurve 1 : Atmung einer Kontrollkultur
« 2 : Veränderung nach Zugabe von 20 Gamma Aluminium
« 3 : « « « « 60 « Blei
« 4 : « « « « 20 « Zinn
« 5 : « « « « 60 « «

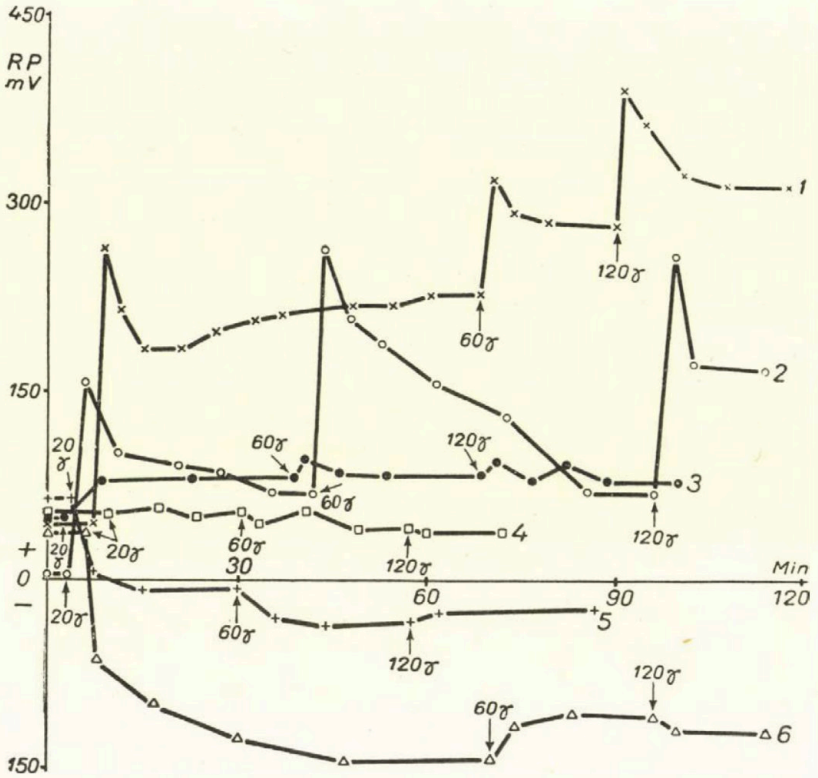

Abb. 3/a. Veränderung des Redoxpotentials von Streptomyces aureofaciens-Schüttelkulturen auf Wirkung verschiedener Metallionen

Kurve	1 :	RP-Veränderung	nach	Zugabe	von	Kupfer
«	2 :	«	«	«	«	Eisen
«	3 :	«	«	«	«	Kadmium
«	4 :	«	«	«	«	Aluminium
«	5 :	«	«	«	«	Vanadium
«	6 :	«	«	«	«	Zinn

Redoxpotential wesentlich beeinflussen. Dies bestätigt die praktische Erfahrung, derzufolge die Aureomycinproduktion in Eisenfermentoren eine geringe ist [4]. Die aus Aluminiumfermentoren herausgelösten Metalle, wie Aluminium, Magnesium und Silikat üben dagegen auf die Atmung und das RP keinen wesentlichen Einfluss aus, so dass es vorteilhafter scheint, die zur Aureomycindarstellung dienenden Einrichtungen aus Aluminium verfertigen zu lassen.

Zusammenfassung

Es wurde die Wirkung verschiedener Schwermetalle auf die Atmung und das Redoxpotential von *Streptomyces aureofaciens*-Kulturen geprüft. Die Messungsergebnisse bestätigen die Erfahrung, dass zur Aureomycinproduktion aus Aluminium hergestellten Fermentoren am besten geeignet sind.

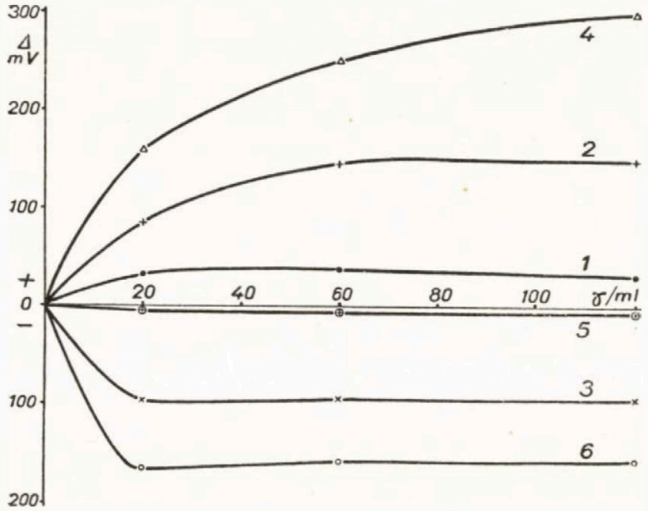

Abb. 3/b. Veränderung des Redoxpotentials von Streptomyces aureofaciens-Schüttelkulturen (Delta mV) auf Wirkung verschiedener Metallionen

Kurve 1: RP-Veränderung nach Zugabe von Kadmium
« 2: « « « « Eisen
« 3: « « « « Vanadium
« 4: « « « « Kupfer
« 5: « « « « Aluminium
« 6: « « « « Zinn

Abbildung 3/b zeigt die auf die Wirkung steigender Mengen verschiedener Metalle in Erscheinung tretenden RP-Veränderungen, aber ohne Berücksichtigung der in Abbildung 3/a enthaltenen vorübergehenden Schwankungen während der Zugabe.

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ВЛИЯНИЕ ТЯЖЕЛЫХ МЕТАЛЛОВ НА ДЫХАНИЕ И РЕДОКСПОТЕНЦИАЛ КУЛЬТУР STREPTOMYCES AUREOFACIENS

Э. Ф. ПЕТТКО, П. КИШШ и А. КРАМЛИ

Резюме

Авторы исследовали влияние различных тяжелых металлов на дыхание и редокспотенциал культур Streptomyces aureofaciens. Результаты измерений подтверждают практический опыт, по которому для производства ауремицина наиболее подходящими являлись ферментаторы, изготовленные из алюминия.

HAEMAGGLUTINATION TEST FOR THE DEMONSTRATION OF C-REACTIVE PROTEIN

By

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In the acute phase of various inflammatory processes a pathological protein known in literature as C-reactive protein (CRP), appears in the blood. It has been named so because it forms a precipitate with the somatic C polysaccharide of the pneumococcus, probably due to a similarity in their molecular configuration.

Depending on the manner in which the bacterial cells are attacked, the pneumococcal C substance is obtained in either of two forms ; using the refrigeration method of TILLET et al. [1], C polysaccharide results, while on treatment with sodium desoxycholate the more polymerized form known as Cx polysaccharide [2]. Whereas human CRP precipitates with both the C and Cx polysaccharides, the protein that appears in rabbits suffering from acute inflammatory disease forms a precipitate with the Cx polysaccharide only [4] ; hence the term, Cx reactive protein.

Conditions of the appearance of CRP and CxRP, their properties, and the factors influencing the precipitation reaction, have been discussed in a number of publications [3, 4, 5, 6].

ANDERSON and MCCARTY have shown [7] that CRP in the blood closely follows the changes in rheumatic fever activity, and that therefore its determination is a useful diagnostic test. Subsequently, several authors have confirmed this observation.

Three methods have been described for the determination of CRP. Chronologically the first, precipitation with pneumococcal C polysaccharide [1]; the second, the capsular swelling reaction of pneumococcus [8] ; the third, precipitation with immune serum prepared in the rabbit against crystalline CRP [9]. A more sensitive modification of the latter reaction is the complement fixation technique with immune serum [14].

Of these reactions it is undoubtedly the third one which is the most sensitive and the most specific ; it appears from the literature, however, that owing to the difficulty in producing CRP in a crystalline form, reactions with the pneumococcal C polysaccharide are more frequently used in clinical practice. The capsular swelling reaction involves a cumbersome procedure and yields uncertain results, therefore it has been discarded everywhere.

The precipitation test as used at present is, however, not a sufficiently quantitative method. Therefore, we set ourselves the aim to elaborate a reaction more suitable for CRP estimation. On adsorbing C polysaccharide on to erythrocytes it has been found that red blood cells sensitized in this manner were agglutinated at a high titre by sera containing CRP.

Materials and methods

1. *Preparation of the somatic C polysaccharide.* R strains of type II, in which there is no capsular polysaccharide are generally used for this purpose. We had no such strain at our disposal, so a strain of type I was taken from the stock of our Institute. This was cultured in serous horse-meat bouillon improved with dextrose and a mixture of various amino acids and vitamins. The cellular sediment of 18-hour broth cultures, twice washed with physiological saline, was invariably used for the preparation of the polysaccharide.

Both the C and the Cx polysaccharides were prepared in the course of the experiments. In preparing the C substance, the cell suspension was alternately frozen with liquid air and then heated, in quick succession 6 to 8 times, while Cx polysaccharide was prepared by treatment with Na-desoxycholate. In the latter method, as much of the Na-desoxycholate (100 mg/ml) dissolved in hot n/100 NaOH was added after cooling to the cell suspension as would bring its concentration to 0,1 per cent. In a water bath at 37° C the cells had dissolved immediately and 2 to 3 minutes later no unimpaired cells could be found in stained preparations.

Of the various methods described for preparing C polysaccharide, the one of McCarty and Avery [6] was adopted in the present experiments; since the original method had been worked out for R strains, it had to be modified. After dissolving the pneumococci the cellular debris was removed and one part of ethyl alcohol had been added to the supernatant; a mass of fibrin-like precipitate was thus obtained which gave no reaction with serum containing CRP. This precipitate was centrifuged and discarded. The subsequent steps were those of the method already described. The end product was re-dissolved in physiological saline, and the purifying process repeated twice. In this manner 10 to 30 mg of polysaccharide were obtained from 1000 ml of pneumococcal culture.

2. *Annular precipitation with C and Cx polysaccharides.* LANCEFIELD's [10] microprecipitation method was used. Of the serum to be tested about 0,1 ml was measured into capillary tubes and 0,1 ml of C or Cx polysaccharide dissolved in saline in a concentration of 0,1 mg/ml was carefully layered upon it. The tubes were placed in a water bath of 37° C and the result was read an hour later.

For the purposes of the haemagglutination reaction described below the sera were inactivated at 56° C for 30 minutes. This process does not affect the precipitation reaction in any manner. At a temperature of —7° C, the sera were found to be preservable for 6 to 8 weeks.

When testing decalcified sera for their precipitation properties, 0,1 ml of a 30 per cent sodium citrate solution was added to 1 ml of the serum.

3. *Precipitation reaction with anti-CRP serum.* This reaction was performed with the method described by ANDERSON and MCCARTY [7], but on reading the results the intensity of the precipitation was disregarded, *i. e.* no different grades of it were established.

4. *Erythrocytes used in the haemagglutination reaction.* Red blood cells from man or from different animal species were washed three times with physiological saline and then placed in a refrigerator for 6 to 8 days; cells older than this were not used. The most commonly used sheep red blood cells were conserved in ALSEVER's solution [11] containing 30 gamma/ml of chloramphenicol; under sterile conditions at +4° C, it was possible to store the erythrocytes in this manner for 6 to 8 weeks.

In titrating the amount of erythrocytes and the concentration of the polysaccharide solution used for sensitizing them, it was intended to determine the lowest polysaccharide concentration capable of sensitizing the greatest amount of erythrocytes by yielding the highest titre with the positive sera. With our polysaccharide preparation and the preserved sheep red blood cells the highest titre was obtained when in an 0,5 per cent polysaccharide solution 5 per cent erythrocytes were suspended, and a 1 per cent sensitized erythrocyte suspension was used for the reaction itself. Sensitization was made by suspending in the 0,5 per cent polysaccharide solution 5 per cent red blood cells and keeping the suspension in the water bath at 37° C for 2 hours, shaking it mildly from time to time. After 2 hours the sensitized erythrocytes were washed twice with physiological saline and then used without delay, as they cannot be stored in the refrigerator for longer than overnight.

5. *The haemagglutination reaction.* In performing this reaction two different procedures were employed. One was to measure the dilutions into tubes 100 mm deep and 10 mm in diameter, and then to add the sensitized erythrocytes. The other was to perform the reaction on the plastic slides described by *Takátsy* [12, 13], using a spiral loop for dilution.

The reaction was performed as follows. Inactivated serum was diluted with physiological saline to 1/25 and 1/37,5 and further diluted in a geometrical ratio to 50, 75, 100, 150, 200, 300, 400, 600, 800, 1200. Adding to each of these serum dilutions an equal amount of the 1 per cent suspension of sensitized erythrocytes, the mixture was placed in a water bath or a thermostat for 2 hours and repeatedly shaken. Then it was transferred to the refrigerator and the results were read according to the distinctness of the edges of the sedimented disc of erythrocytes after 4 to 8 hours, or eventually overnight.

Results

1. *Properties of the C and Cx polysaccharides prepared.* The polysaccharide preparation obtained in the final purifying step was a snow-white amorphous powder, which dissolved in sterile water within a few minutes to a crystal-clear solution. With ninhydrin it gave a positive reaction. Analysed chromatographically, it revealed three components with both phenol and butanol solvents. Subjected to hydrolysis with hydrochloric acid, no increase in the number of mobile components was observed. Reaction with pentose was negative. It appears, then, that the polysaccharide prepared by us contained no contamination by nucleic acid or protein. The only foreign substances in it were three components (amino acids) reacting with ninhydrin.

With these preparations we have succeeded in reproducing the experiments described in the literature [4]. If, namely, rabbits are experimentally infected, CxRP appears in their blood in the acute phase of the disease, and this protein reacts solely with Cx polysaccharide. The precipitation reactions performed with the different preparations are presented in Table I.

Table I

Precipitation reaction with C and Cx polysaccharide preparations

Preparation	Human serum No. 6	Rabbit serum No. IV
C_1	+	—
C_2	+	—
Cx_1	+	+
Cx_4	+	+
Cx_5	+	+
Cx_6	+	+
Cx_7	+	+
Cx_8	+	+

2. *Properties of the haemagglutination reaction.* As already mentioned the precipitation reaction was not considered to be suitable for the demonstration

of CRP, partly because it is not sufficiently sensitive, partly because it gives no quantitative results. On this account, it was attempted to adsorb the polysaccharide on erythrocytes and determine the CRP by means of the haemagglutination reaction.

For this purpose, the demonstrability of the CxRP appearing in the serum of rabbits experimentally infected with LANCEFIELD's A-group haemolytic streptococcus, was compared by the classical precipitation method and the new hemagglutination test. Three rabbits were infected intracutaneously with 0,2 ml of an 8-hour bouillon culture of haemolytic streptococcus, and

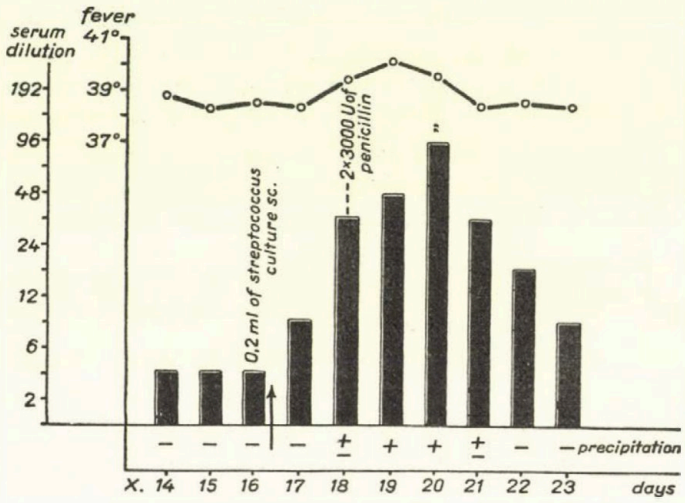

Fig. 1. Changes in the Cx-reactive protein in an experimentally infected rabbit (female, weighing 3,5 kg)

thereafter blood was taken daily from their auricular vein. The CxRP level was determined each day with both precipitation and haemagglutination. One animal died on the third day, the other two presented approximately identical symptoms. The data referring to one of the experimental animals are shown in Fig. 1.

The animal experiments having brought proof that with haemagglutination it is possible to follow the changes in the CRP level, the next step was to study the factors influencing the reaction.

The influence of temperature is shown in Table II.

It can be seen from Table II that the reaction occurs over a wide range of temperature, wherefore incubation in a water bath or a thermostat can be dispensed with.

The following Table III shows the effect of the pH.

The pH of the saline used in these experiments was adjusted with 0,03 M Sörensen buffer. The tabulated results show that haemagglutination is not

Table II

Effect of temperature upon haemagglutination
(Human serum No. 47; preparation Cx_8)

	Serum dilution								
Temperature	1 : 100	1 : 150	1 : 200	1 : 250	1 : 300	1 : 350	1 : 400	1 : 500	Control
+ 4° C	+	+	+	+	+	+	—	—	—
+ 18° C	+	+	+	+	+	+	+	—	—
+ 37° C	+	+	+	+	+	+	+	—	—

particularly sensitive to changes in pH. Results were the same between pH 6 and 8, and from this it follows that there is no need for the use of a buffer in the reaction.

Table III

Effect of pH on hemagglutination
(Human serum No. 16; preparation Cx_4)

pH (0,03 M Sörensen buffer)	Serum dilution								
	1 : 150	1 : 150	1 : 200	1 : 250	1 : 300	1 : 350	1 : 400	1 : 500	Control
5,36	+	+	+	+	+	+	—	—	—
5,64	+	+	+	+	+	—	—	—	—
6,03	+	+	+	+	+	+	—	—	—
6,40	+	+	+	+	+	+	—	—	—
6,78	+	+	+	+	+	+	+	—	—
7,20	+	+	+	+	+	+	+	—	—
7,66	+	+	+	+	+	+	—	—	—
7,98	+	+	+	+	+	+	+	—	—

The haemagglutination reaction can be greatly influenced by the ionic environment of the erythrocytes. For this reason haemagglutination reactions were performed with NaCl, KCl and $CaCl_2$ solutions of different molar concentration. In these solutions an adequate dose of dextrose served to secure isotonia. The sensitized erythrocytes were washed with similar solutions. Results are shown in Tables IV and V.

In $CaCl_2$ environment the erythrocytes were found to agglutinate spontaneously.

It was further examined what kind of erythrocytes were best suited for the purposes of haemagglutination reaction.

Table IV

Haemagglutination in NaCl environnement
(Human serum No. 47; Preparation Cx_4)

Molar concentration of NaCl	Serum dilution								
	1 : 100	1 : 150	1 : 200	1 : 250	1 : 300	1 : 350	1 : 400	1 : 500	Control
0,025	+	—	—	—	—	—	—	—	—
0,0375	+	+	—	—	—	—	—	—	—
0,050	+	+	+	+	—	—	—	—	—
0,075	+	+	+	+	+	+	—	—	—
0,100	+	+	+	+	+	+	—	—	—
0,125	+	+	+	+	+	+	+	—	—
0,150	+	+	+	+	+	+	—	—	—
0,175	+	+	+	+	+	—	—	—	—
0,200	+	+	+	+	+	—	—	—	—
0,250	+	+	+	—	—	—	—	—	—

Table V

Haemagglutination in KCl environment
(Human serum No. 47; preparation Cx_4)

Molar concentration of KCl	Serum dilution								
	1 : 100	1 : 150	1 : 200	1 : 250	1 : 300	1 : 350	1 : 400	1 : 500	Control
0,025	—	—	—	—	—	—	—	—	—
0,0375	+	—	—	—	—	—	—	—	—
0,050	+	—	—	—	—	—	—	—	—
0,075	+	+	—	—	—	—	—	—	—
0,100	+	+	—	—	—	—	—	—	—
0,125	+	+	+	+	—	—	—	—	—
0,150	+	+	+	+	+	—	—	—	—
0,175	+	+	+	+	—	—	—	—	—
0,200	+	+	+	+	—	—	—	—	—
0,250	+	+	+	+	+	—	—	—	—

It can be seen from Table VI that guinea pig red blood cells gave the highest titre. In practice, it is nevertheless more expedient to use sheep erythrocytes because those of the guinea pig are less readily available in sufficient

Table VI

Haemagglutination reaction with different erythrocytes
(Human serum No. 73 ; preparation Cx_8)

Erythrocyte	Serum dilution									
	1 : 100	1 : 150	1 : 200	1 : 300	1 : 400	1 : 600	1 : 800	1 : 1200	1 : 1600	Control
Human «O»	+	+	+	—	—	—	—	—	—	—
Chicken	+	+	+	+	—	—	—	—	—	—
Sheep	+	+	+	+	+	+	?	—	—	—
Guinea pig	+	+	+	+	+	+	+	+	—	—
Rabbit	+	+	+	+	+	+	+	+	+	+

quantities. Results obtained with rabbit red blood cells were found to be aspecific ; the non-sensitized controls were also agglutinated by the serum tested.

Finally, it was examined whether haemagglutination occurs in a Ca-free medium, remembering that no precipitation occurs if the serum has been decalcified with citrate or oxalate. The data presented in Table VII show, however, that decalcification does not prevent the reaction.

Table VII

Precipitation and haemagglutination reaction before and after decalcification
(Human sera ; preparation Cx_4)
Decalcification with 30% sodium citrate

Serum	Precipitation	Haemagglutination	Precipitation	Haemagglutination
	before decalcification		after decalcification	
6.	+	1 : 400	—	1 : 400
11.	+	1 : 400	—	1 : 400
16.	+	1 : 400	—	1 : 300
20.	+	1 : 600	—	1 : 600
31.	+	1 : 300	—	1 : 300
32.	+	1 : 400	—	1 : 300
33.	+	1 : 400	—	1 : 400
38.	+	1 : 400	—	1 : 400

3. *Comparison of the haemagglutination method with the precipitation reactions in use.* Two series of experiments were performed in order to compare our new method with the precipitation reaction induced by CRP antiserum by C polysaccharide, respectively. The results of these comparative experiments are given in Table VIII.

Table VIII shows that with CRP antiserum positive precipitation reactions were obtained whenever the titre of haemagglutination was 1 : 150 or higher. The same was found to apply to the C polysaccharide but only for titres of

Table VIII

Comparison of the haemagglutination method with the usual precipitation reactions

Haemagglutination titre of sera	On precipitation with anti-CRP sera precipitate yielded	On precipitation with C-polysaccharide precipitate yielded
1 : 800	2/2*	6/6
1 : 600	n. t.	2/2
1 : 400	5/5	4/4
1 : 300	3/3	6/6
1 : 200	3/3	5/6
1 : 150	2/2	2/6
1 : 100	5/7	3/11
1 : 75	1/2	0/14
1 : 50	1/3	0/5
1 : 50 >	2/4	0/3

* Numerator, number of sera giving positive reaction—Denominator, number of sera tested

or above 1 : 200. Comparing these results with clinical observations it can be claimed that in dilutions of 1 : 150 or higher, the hemagglutination reaction is invariably positive.

4. *Clinical applicability of the haemagglutination method.* The method has now been followed by us for 3 months. In patients suffering from various conditions at the Department of Paediatrics the CRP titre had been continuously referred to the clinical picture, the course of the temperature, the sedimentation rate, and the changes in the serum protein fractions. So far, 300 determinations have been performed and their results are on the whole in agreement with those of CRP estimations made with other methods.

When applying the haemagglutination method in clinical practice it must, however, be borne in mind that sheep erythrocytes sensitized with the pneumococcal polysaccharide are also agglutinated by many normal sera, although the titre of this agglutination never exceeds 1 : 75. At the same time, in none of our patients suffering from inflammatory diseases has a titre of under 1 : 150 been encountered. For this reason, in evaluating the reaction titres of 1 : 75 were regarded as normal and agglutinations in a dilution of 1 : 100 as uncertain. No agglutinations with a titre under 1 : 150 were regarded as positive.

According to the literature, high CRP levels are found in a variety of inflammatory diseases, such as tonsillitis, mastoiditis, tendovaginitis, stomatitis,

meningitis, rheumatic fever, purulent meningitis, and appendicitis. In Fig. 2 the changes in the level of CRP as determined by the quantitative haemagglutination method are illustrated in two children one of whom suffered from rheumatic polyserositis and the other from mastoiditis. The data include changes in the temperature, sedimentation rate, alpha and gamma globulin, and CRP level.

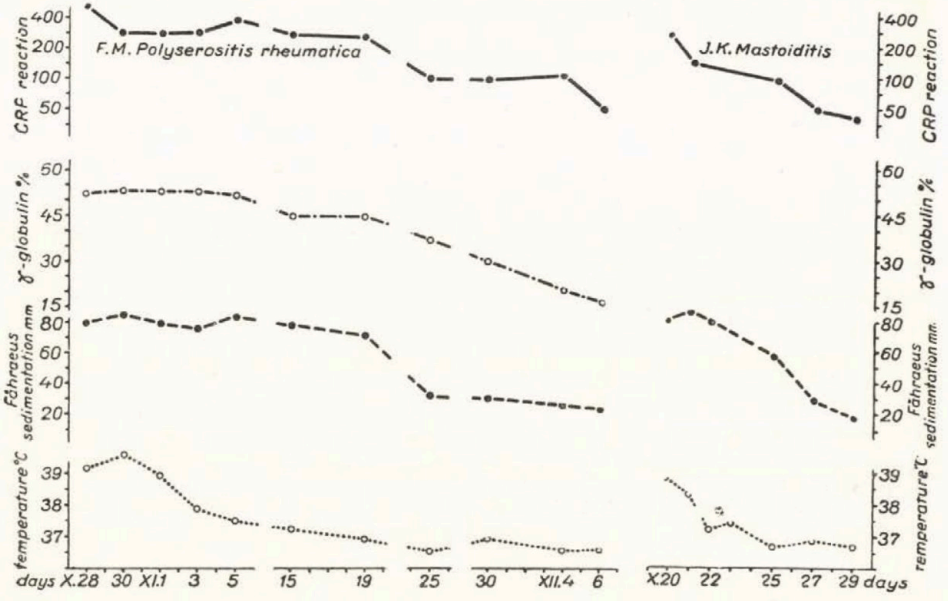

Fig. 2. Comparison of the haemagglutination reaction of the generally used laboratory methods for the indication of inflammatory processes

Changes in the CRP level were found similar in other inflammatory diseases.

A high sedimentation rate was accompanied by agglutination under 1 : 75, *i. e.* a negative result was obtained in leukemia, genuine nephrosis, and reticulosarcoma. This too appears to be in agreement with the general conception concerning the origin and appearance of CRP.

Discussion

Our experiments aimed at the elaboration of a quantitative method for determining CRP by the use of pneumococcal C polysaccharide, a substance relatively easy to prepare. The haemagglutination method described above can easily be performed in clinical laboratories, is not sensitive to changes in temperature and in pH and yields quantitative results that are easily estimated.

Only crystal clear sera are suitable for the usual precipitation reactions, and in clinical practice they are not always easy to prepare. While the purifying by means of Seitz filtering requires a fairly large amount of serum, not more than 0,2 ml of a not necessarily crystal clear translucent serum are needed to perform the reaction elaborated by us.

Since the haemagglutination reaction cannot be regarded as positive unless its titre is at least 1 : 150, it is not much more sensitive than precipitation with the C polysaccharide. Again, our comparative tests have shown that sera which agglutinated sensitized erythrocytes in dilutions up to 1 : 200 gave invariably positive precipitations with the C polysaccharide as well.

Haemagglutination occurring in lower dilutions cannot be regarded as undoubtedly positive because red blood corpuscles sensitized with the C polysaccharide are agglutinated by many normal sera also in dilutions under 1 : 75. These agglutinations at a low titre must in our opinion be due to antibodies present in most healthy persons, in contradistinction to the somatic polysaccharides of the streptococcus group, the antigenic structure of which is closely affined.

A quantitative estimation of the results obtained by the haemagglutination reaction is sometimes difficult because haemagglutination occurs also in decalcified media. There are, namely, cases in which it has to be decided whether the positive result is due to CRP or an antipneumococcal C polysaccharide antibody. In precipitation reactions this point is easily decided by decalcification of the serum, as in a Ca-free medium there is no observable precipitation.

The experience so far accumulated in testing patients' sera with the haemagglutination reaction seems to be promising. Individual results obtained could always be reproduced and, if referred to the clinical picture, the reaction reassuringly indicated the changes that had occurred in the CRP level. There seems to be reason to believe that the haemagglutination reaction might become a useful procedure in laboratory tests.

Summary

1. One of the methods to obtain the C polysaccharide present in pneumococci (the one evolved by McCarty and Avery) has been so modified as to be suitable for the preparation of the C substance from S strains as well.

2. With the C polysaccharide prepared, sheep red blood cells were sensitized, and with these sensitized erythrocytes a haemagglutination reaction has been elaborated to determine the CRP. The reaction can be elicited over a wide range of temperature and pH, and also in decalcified media. Sheep red blood cells are best suited for the purposes of haemagglutination.

3. The haemagglutination reaction has been compared, in respect of sensitivity and specificity, with the precipitation methods in general use.

4. The applicability of the new haemagglutination procedure has been demonstrated in some clinical cases.

*

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ГЕМАГГЛЮТИНАЦИОННЫЙ МЕТОД ДЛЯ ВЫЯВЛЕНИЯ С-РЕАКТИВНОГО ПРОТЕИНА

К. ГАЛ и М. МИЛЬТЕНЬИ

Резюме

Авторы поставили перед собой цель выработать количественный способ выявления С-реактивного протеина с помощью полисахарида целлюлярного пневмококка, т. е легко получаемым веществом. В первой части работы они видоизменили выработанный до настоящего времени метод получения целлюлярного полисахарида и сделали его пригодным для выявления полисахарида из штаммов S. Полученным таким образом полисахаридом сенсибилизировали эритроциты овец и определили, что сыворотки содержащие С-реактивный протеин агглютинировали эти эритроциты в высоком титре. Во второй части работы авторы определили свойства этой реакции агглютинации и подробно изложили влияющие на реакцию факторы. Применимость реакции гемагглютинации демонстрировали несколькими клиническими примерами.

ON THE PROTEASE AND UREASE ENZYMES OF STREPTOMYCES GRISEUS

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In the laboratory and in the experimental production unit of the above Institute, during submerged fermentation of *Streptomyces griseus*, systematic studies have been made of the behaviour of certain components of the medium, so, for instance of that of soluble nitrogen. The results of these studies had stimulated us to approach the problem from a different angle and it could be shown that under the experimental conditions employed the microorganism produces a potent exocellular protease, which proved to be precipitable with acetone or with methanol and thus could be rendered suitable for closer examination.

The other enzyme demonstrated to be present in submerged cultures was urease. This enzyme will be dealt with in Part II. of this paper.

1. The exocellular protease of Streptomyces griseus

The main protein source of the medium was 2 per cent soy bean meal' insoluble in water. Other components of the medium included 0,4 per cent corn steep liquor; 2 per cent glucose or 2,5 per cent starch, and 0,25 per cent sodium chloride. The Streptomyces griseus had been cultured by DR. LOVREKOVICH, by irradiating a strain obtained from a foreign country.

In the course of fermentation the mycelium became fully developed in 48 hours following inoculation, so that it could be anticipated that the amount of soluble nitrogen was markedly decreased in the medium. The values obtained were, however, highly variable, as it is shown in Table I.

It has been suggested that this phenomenon is due to the fact that under submerged deep-fermentative conditions the Streptomyces produces an exocellular protease, which splits off from the insoluble soy bean meal small molecular, N-containing, soluble substances and thus the nitrogen built in into the developing mycelium will only slightly modify the amount of N in the medium.

In the first experiments the effects of the above enzyme had been demonstrated by the following method. To 30 ml of sterile fermentative medium in

Table I

Changes in the organic nitrogen content in glass fermentor

No.	After	mg per 100 ml 0	1	2 days
251/2		—	67,3	69,3
253/4		65,4	60,9	61,6
255/4		94,3	58,0	61,3
259/2		55,0	50,5	58,0
261/1		66,1	59,9	60,9
261/3		69,2	61,3	77,8
265/6		74,9	55,0	69,1
524/2		63,8	48,2	61,7
527/1		—	57,0	55,1
528/4		—	88,8	93,5
280/1		48,7	29,1	47,1
534/2		80,4	79,8	78,1
534/1—4		—	76,4	64,3
535/2		71,3	64,9	59,4
535/4		95,3	52,2	77,2
536/2		72,0	62,3	73,8
Average		71,3	60,7	68,2

an Erlenmeyer flask were added 15 ml of a mycelium-free centrifugate of fermentation fluid of varying age, and 10 drops of toluol.

A sample was then taken from the flask to determine soluble nitrogen. Subsequently the flask was placed into an incubator of 28° C and sampled for estimating its soluble nitrogen content after 24 hours, or, in some instances, after 48 hours. The results are shown in Table II.

In 7 experiments 24-hour fermentation fluid was used. The level of soluble N increased in 6 cases, by 17 per cent on the average. In each one of the ten fermentation fluids examined after 48 hours, the value of soluble N was found to have increased by an average of 24,9 per cent. This means that exocellular protease was produced by the microorganism during the first 2 days of fermentation already.

Adding to the fermentation fluid acetone or methanol at a ratio of 1 to 5, a copious precipitate was formed. Drying this in a vacuum exsiccator, a greyish-brown, non-hygroscopic powder resulted. The yield was 0,5 to 0,8 g of powder from 100 ml of fermentation fluid.

In order to compare the activity of the crude enzyme powder with the crude pancreas powder (pancreatin) official in the Hungarian Pharmacopoeia, the powder was first tested by the method prescribed for pancreatin in the

Table II

Increase of soluble nitrogen in the medium under the influence of exocellular protease

No.	Duration of fermentation (days)	Soluble nitrogen in mg per 100 ml		
		0	24	48 hrs.
528/4	1	81,9	87,6	104,5
280/5	1	82,0	86,0	—
292/2	1	54,5	50,6	—
292/5	1	65,8	73,9	—
534/2	1	69,7	112,0	137,0
534/1	1	90,2	110,6	102,9
F_3	1	76,4	88,4	—
Average		74,3	87,0 (17,0%)	
259/1	2	61,0	100,6	123,0
265/6	2	66,6	86,5	—
528/4	2	81,9	99,3	—
280/1	2	62,0	94,0	—
F_1	2	84,0	89,5	—
292/22	2	50,7	80,1	—
F_2	2	88,7	96,6	—
534/2	2	78,6	99,1	106,9
534/4	2	77,0	103,8	101,3
Average		74,5	93,1 (24,9%)	

IV., then in the V. Pharmacopoeia Hungarica. The essence of the method described in the latter Pharmacopoeia is that digestion in 30 minutes of a 0,2 per cent casein solution in a borate buffer is examined at different enzyme concentrations in a water bath of 40° C. The enzyme is acceptable if 1 mg of it is capable of digesting at least 25 mg of casein. The enzyme powders derived from Streptomyces griseus met this requirement in every test, and, not infrequently, they even exceeded it. In one case a 5 months old sample of powdered enzyme was tested for activity and even this sample proved to have the desired potency.

After maintaining 20 mg of the enzyme dissolved in 100 ml of distilled water or of the above buffer medium at 60° C for 30 minutes (in a water bath), it has lost its potency to digest casein. If, however, a casein-enzyme mixture, and not the pure enzyme had been kept at 60° C for 30 minutes, casein digestion was not weaker than at 40° C. It appears that dissolved casein and its degra-

dation products protect the enzyme against the otherwise destroying effect of exposure to 60° C.

The exocellular protease effect of Streptomyces griseus was tested not only against casein or soy bean meal, but also against peptone and fibrin. The technique employed in one of the experiments with peptone was the following. Into each Erlenmeyer flask were measured 5 ml of a 4 per cent solution of Witte peptone and 5 ml of the solution containing 5 mg of the enzyme. The control flasks contained 5 ml of distilled water instead of enzyme. After adjusting the pH to 7,5 with N NaOH and adding 0,5 ml of toluol, the flasks were incubated at 37° C for 20 hours. The lytic action was determined by the formol titration method in the modification of recommended by NORTHROP [1]. The amino nitrogen content of the control peptone solution was 3,7 mg, as compared to the 7,4 mg value found in flasks containing enzyme.

Fibrin digestion was studied by the following method. Into each 25-ml Erlenmeyer flask had been added 1 ml of a 0,2 mol. borate buffer (pH 8,32) and, dissolved in 2 ml of distilled water, 2,5, 5, and 10 mg of enzyme, respectively. The control flasks contained 2,0 ml of distilled water without enzyme. Also added to each flask had been 6 ml of 1 per cent bovine fibrinogen, and five minutes later 1 ml (8 U) of thrombin. A firm fibrin coagulate was formed. The flasks were then incubated at 37,5° C. After 20 minutes the content of the flask containing 10 mg of enzyme was liquid ; liquefaction occurred in the 5 mg enzyme flasks after 30 minutes, and in those containing 2,5 mg of the enzyme after 70 minutes.

The protease of Streptomyces griseus has a marked coagulative effect on milk. In a concentration of 0,5 mg/ml (total fluid volume) it coagulated milk in 15 minutes at 40° C, as determined in tests with various concentrations of enzyme.

The optimum pH was also determined. Four g of casein of good quality, 4 ml of N sodium hydroxide and 90 ml of distilled water in an Erlenmeyer flask were thoroughly shaken for 1 hour, made up to 100 ml and boiled. In the lower pH ranges the buffer used was 0,2 m Na_2HPO_4 and 0,1 m citric acid, as recommanded by MCILVAINE [2]. In higher p_H ranges mixtures of 0,1 ml of glycine and 0,1 m NaOH were employed, according to SÖRENSEN [2]. The enzyme powder was homogenized in a small volume of casein solution in a grinding mortar and diluted to contain 1 mg of enzyme in each ml of the casein solution. Into each Erlenmeyer flask were measured 3 ml of the latter homogenizate, and 2 ml of buffer, prepared in advance. The pH of the buffer varied from flask to flask. There were two control flasks to each enzyme-containing one ; they contained all the above ingredients, but no enzyme. One of the two control flasks served the purpose of electrical pH determination. Incubation occurred at 37° C.

Usually 24 hours later the contents of the flasks were tested for enzymic activity by SÖRENSEN's formol titration method, using the technique described

by NORTHROP. To each flask were added 1 ml of a 0,1 N NaOH and one drop of 0,5 per cent phenolphthalein, except the flasks used for pH determination. After having determined the pH, into one of the flasks were added 1 ml of formaldehyde and, instead of 1 ml, 2 ml of 0,1 N NaOH and 1 drop of a 0,5 per cent phenolphthalein solution. The enzyme-containing and the control flasks alike were titrated with N/50 NaOH against the red colour of the latter flask containing 0,1 per cent phenolphthalein. The difference in titre between each enzyme-containing flask and the corresponding control flask give the activity for the actual pH in terms of ml of N/50 NaOH. The optimum pH was in the single tests 7,94; 8,34; 8,57; 7,94; 8,44. The activity curve plotted in an experiment of this kind is presented in Fig. 1.

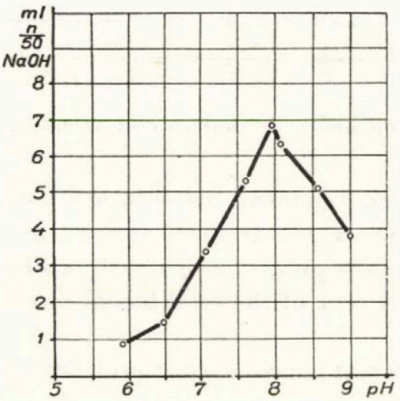

Fig. 1. Determination of the optimum pH of Streptomyces griseus protease

The precise method of estimating the activity of the exocellular protease of Streptomyces griseus was as follows. Into each 50 ml Erlenmeyer flask were added 5 ml of 4 per cent casein, 2 ml of 0,2 m phosphate buffer of pH 7,73, and 3 ml of the solution containing 4,0 mg of the enzyme. The flasks were placed in a water bath of 39° C and titrated by the formol method, at 0,30, 60 and 90 minutes in two flasks at each point of time. At the beginning, total nitrogen was also estimated. The results are shown in Table III.

Table III

Estimation of protease activity. 39° C, 4 per cent casein

Time in minutes	0	30	60	90
Amino N in mg	2,71	3,01	3,29	3,56
Total N in mg	27,65	—	—	—
$K \cdot 10^3 = \frac{1}{t} \frac{a}{a-x}$	—	0,175	0,170	0,169

The uniform value of the reaction rate constant in Table III. indicates that under the experimental conditions specified digestion of casein proceeded as a reaction of first order.

The result of a 23-hour casein digestion is shown in Fig. 2.

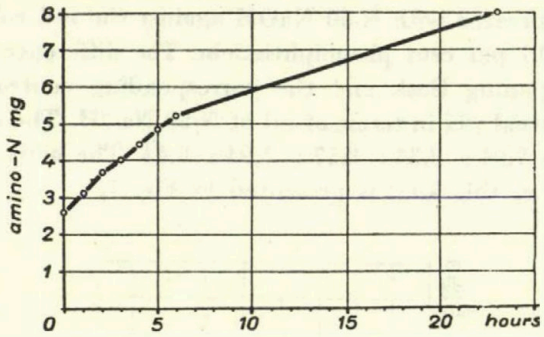

Fig. 2. Casein digestion under the influence of Streptomyces griseus protease

The experimental arrangement was identical with that described above, except that incubation was made at 36° C and not at 39° C. The slowing rate of casein digestion with time progresses is clearly visible from the shape of the curve. In 23 hours 32 per cent of the total bound nitrogen were converted into free amino nitrogen.

The effect of different enzyme concentrations on casein digestion was examined partly by means of estimating nitrogen after precipitating the protein

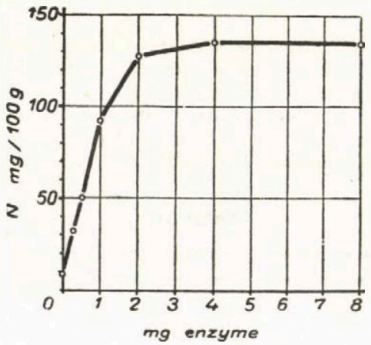

Fig. 3. Effect of Streptomyces griseus protease concentration on the digestion of casein

with trichloracetic acid, partly by the formol titration method. Into each 50-ml Erlenmeyer flask were placed 5,0 ml of 4 per cent casein, 2 ml of 0,2 m borate buffer and 3 ml of the enzyme-containing solution, in which there were 0,25,

0,5, 1,0, 2,0, 4,0, 8,0 and 16,0 mg of enzyme, respectively. The pH of the mixture was 8,4. After 18 hours 10,0 ml of 10 per cent trichloracetic acid were added to each tube to precipitate protein. In the flasks containing 4 mg or more of enzyme no precipitate was formed. The results of N estimations in filtrates are shown in Fig. 3.

The curve in Fig. 3. reveals that with 0,25, 0,5 and 1,0 mg of enzyme the rate of casein digestion was directly proportional to the enzyme concentration, inasmuch as 0,25 mg increased the amount of non-protein-nitrogen by 21,6 mg, 0,5 mg by 40,79 mg and 1,0 mg of enzyme by 84,09 mg, respectively. Four mg of enzyme practically degraded during the experiment already all the protein of casein to products not precipitable with trichlor acetic acid.

In the formol titration series the effect on casein digestion of various enzyme concentrations was examined in non-buffered medium. Into each 50 ml Erlenmeyer flask were measured 5 ml of 4 per cent casein and 5 ml of the enzyme solution. The pH was adjusted to 7,0. In 22 hours free amino nitrogen titratable with formol was increased by 2,8 mg in the 2 mg enzyme flasks, by 4,0 mg in the 4 mg enzyme flasks and by 5 mg in the 8 mg enzyme flasks. In other words, 2 mg of enzyme increased the breakdown by 2,8 mg, and this effect was further increased by only 1,2 mg by 4 mg of enzyme, and by 2,4 mg by 8 mg of enzyme.

In such a 20 or 24-hour casein digestion flask the biuret reaction results in the appearance of an intensive red colour. After precipitation with trichloracetic acid, the reaction in the filtrate gives the red colour characteristic of peptones, while the undigested casein solution alone becomes of a violet colour. In such digests no tryptophane can be detected with fresh bromine water.

In an attempt to determine whether or not the protease of *Streptomyces griseus* contains dialysable co-enzyme, experiments were made in cellophane sacks. 20 mg of enzyme were dissolved in 10 ml of distilled water ; 0,5 ml of toluol was added and the solution was dialyzed once against 3 litres of distilled water for 25 hours, and on another occasion against running water for 42 hours. The enzyme solution so treated was then examined for its casein digestive activity, parallel with a control test with non-dialyzed enzyme. In one case the amino nitrogen content obtained with the dialyzed enzyme was 2,4 per cent less ; in the other 4,0 per cent less, than that obtained with the non-dialyzed enzyme. Thus, the experiments brought no evidence indicating the presence of dialyzable co-enzyme.

In other experiments the effect on casein digestion of ferrous sulphate, manganese sulphate H_2S, cysteine, serum, and prussic acid, respectively, was tested. In these experiments buffer-free media were used, like in the studies on bacterial proteinases by MASCHMANN [3]. 5 ml of 4 per cent casein and enzyme dissolved in 3 ml distilled water were used, while the volume of the potential activator or inhibitor was 2 ml. The enzymic solution was usually

incubated together with the test substance for 15 or 30 minutes in a 40° C water bath. Serum or cysteine were not incubated previously. HCN was obtained from chilled KCN solution by treatment with N HCl. The initial pH was adjusted to 7,5 with N/*10* NaOH. Digestion usually lasted for 20 or 23 hours and progress was determined by formol titration. The results are summarized in Table IV. In general, all the subtances tested more or less inhibited the digestion of casein by protease. It was only cysteine at a concentration of 15 mg/ml that showed a very slight, almost non-appreciable increase (2,6 per cent), but a concentration of 30 mg/10 ml had already an inhibitory effect.

Table IV

Effect of different substances on protease activity

Substance	Quantity mg/10 ml	Incubation time min.	Quantity of enzyme mg/10 ml	Related activity Control: 100 per cent
Ferrous sulphate	4,0	15	5,0	83,6
« «	2,5	15	5,0	92,1
« «	1,25	15	5,0	94,4
H_2S	1,5 ml saturated solution	15	5,0	86,8
H_2S	0,5 ml saturated solution	15	5,0	90,1
Cysteine HCl	15,0	0	5,0	102,6
« «	30,0	0	5,0	92,4
Manganese sulphate $1/2H_2O$ ..	7,5	15	5,0	96,8
« « $1/2H_2O$ ..	30,0	15	5,0	92,2
HCN	0,1 mol	15	2,0	71,9
HCN	0,01 mol	15	2,0	80,4
Cat serum	2,0 ml	0	2,0	44,2

2. On the urease enzyme of Streptomyces griseus

IWANOFF [1] has reported that *Aspergillus niger*, *Rhizopus nigricans*, and other low fungi produced ample amounts of urea in the mycelium when grown in media containing peptone and salts, but no or very little carbohydrates. Some of the urea produced was detectable in the medium, too. On adding carbohydrate to the medium urease was produced and the urea disappeared. In the hope that a correlation between carbohydrate content and urea production will be detectable in the course of the fermentative cultivation of *Streptomyces griseus* and that on the basis of urea estimations conclusions can

be drawn whether the amount of carbohydrate in the microorganism is sufficient, samples of fermentation fluid taken at different points of time during fermentation were tested for their urea content.

Urea was estimated by the method of VAN SLYKE and CULLEN [2]. Since no urea could be demonstrated in any of the mycelium-free fermentation fluids, the experiments will not be described in detail.

Since it could be assumed that the absence of urea is due to a breakdown by urease present in the culture of Streptomyces griseus, cultures on Christensen medium [3] were examined for the presence of urease.

Urea-containing Christensen medium and, as a control, urea-free slant agar were inoculated with Streptomyces griseus spores. As control microorganisms, *Staphylococcus aureus* (known to give a positive urease reaction), and *Salmonella typhi* (urease-negative) were grown in these same media. The media inoculated with Streptomyces griseus were incubated at 28° C, while with other organisms the temperature of incubation was either 37° C, or 28° C.

6 to 7 hours following inoculation circumscribed spots of a reddish colour and 1 to 2 mm in diameter, appeared in the tubes containing Streptomyces griseus. 8 hours after inoculation these areas were increased in diameter and, after standing overnight, an intensive reddish-purple colour has developed everywhere in the tubes. The ureafree tubes, as well as those containing *Salmonella typhi* remained yellow. The tubes with *Staphylococcus aureus* showed a reaction comparable to that seen in the tubes with Streptomyces griseus.

This experiment was reproduced with several strains grown by DR. LOVREKOVICH (Budapest) and the results obtained were similar to that described above. A strain of Streptomyces griseus obtained from PROF. IVÁNOVICS (Szeged), and 2 others from the German Democratic Republic also gave positive reactions, although as late as 48 hours after inoculation.

Thus, in each of the Streptomyces griseus cultures grown on CHRISTENSEN medium the presence of urease was detectable.

In order to determine whether the urease so demonstrated was the product of adaptative enzyme production induced by addition to the medium of urea or urease would be produced physiologically in the course of cultivation in the fermentor and would thus be involved in the nitrogen metabolism of the microorganism, extracts prepared from mycelia of Streptomyces griseus fermentor cultures were tested for their urease activity. Also tested for urease activity were mycelium-free samples of fermentation fuid.

The method used was the following. Mycelium-containing liquid from the fermentors was centrifuged, 7 to 10 g of the mycelium so obtained was homogenized with glass powder by grinding for 10 minutes. After adding 20 ml of a pH 6,1 phosphate buffer, the mycelium was extracted at room temperature by frequent mixing and grinding for 1 hour. After that the liquid was separated from the mycelium by centrifugation. The extract obtained was divided into

two equal parts and added to two central tubes of two apparatuses used for estimating urea. To one of the central tubes 30 mg of urea were also added. The other tube served as a control for NH_3 estimation. Two hours later potassium carbonate was added to both tubes and the ammonia content was estimated. In case of a positive urease effect the urea-containing tube contained more ammonia than the urea-free tube.

According to a different technique, to mycelium homogenized with glass powder acetone was added and then 1 g of this acetonedried powdered mycelium was extracted with the pH 6,1 phosphate buffer and tested for urease activity as described above. To the mycelium-free liquid centrifugate was added urea (30 mg to 10 ml) and after 2 hours of incubation it was examined for urease activity against a 10 ml control sample, as described above.

The results for mycelium extracts are shown in Table V.

Table V

Urease activity of mycelial extracts

Date	Mode of extraction	Urea—N + + ammonia—N mg per 100 ml	Ammonia—N mg per 100 ml	Urea—N mg per 100 ml
Sept. 7	Fresh mycelium with phosphate buffer	54,6	6,2	48,4
Sept. 14	« « « « «	58,5	15,4	43,1
Sept. 14	« « « « «	58,2	19,9	38,3
Sept. 21	« « « « «	23,6	16,8	6,8
Sept. 27	« « « « «	20,2	2,9	17,3
Oct. 4	« « « « «	17,1	9,8	7,3
Sept. 7	Fresh mycelium with distilled water	18,3	6,6	11,7
Aug. 31	Aceton powder with phosphate buffer	7,3	2,5	4,8
Sept. 1	« « « « «	12,9	7,7	5,2
Sept. 1	« « « « «	14,2	5,7	8,5
Sept. 3	« « « « «	12,4	7,7	4,7
Sept. 10	« « « « «	7,0	3,0	4,0

As it can be seen in Table V each of the mycelia tested exerted urease activity. In general, the urease activity of aceton powders was lower than that of fresh phosphate buffer extracts. In 3 tests the urease activity was so high that the liberated ammonia had bound the total of 20,0 ml N/50 HCl used. All the mycelia included in Table V were 6 days old taken at the conclusion of the fermentation.

In 3 cases urease activity was determined in 2-days old and 6-days old mycelia from the same fermentation. The results in the 2-days old specimen were as follows ; Sept. 16 1,9 mg ; Sept. 23 9,8 mg ; and Sept. 30 12 mg. The

corresponding 6-day values are shown in Table I (the values on September 21, 27 and October 4). Thus, in all three cases the 2-day specimen already possessed urease activity, in 2 cases lower and in 1 case higher than at the conclusion of the fermentation.

The results obtained with mycelium-free liquid centrifugates were far less uniform than those yielded by the mycelium extracts, as shown in Table VI.

Table VI

Urease activity of the mycelium-free medium

Date	Duration of fermentation days	Urea-N + + NH_3-N mg per 100 ml	Ammonia-N mg per 100 ml	Urea -N mg per 100 ml
Aug. 28	6	59,6	12,3	47,3
Sept. 7	6	24,2	11,0	13,2
Sept. 13	6	24,9	19,4	5,5
Sept. 16	2	3,64	1,68	1,96
Sept. 21	6	32,4	32,8	0,4
Sept. 23	2	5,6	2,5	3,1
Sept. 27	6	4,6	4,5	0,1
Sept. 30	2	1,8	1,5	0,3
Oct. 4	6	22,2	13,5	8,7

In 6 out of 9 fresh centrifugates a varying urease activity could be demonstrated, while in 3 there was no enzymic activity. Also, when the positive centrifugates were tested 24 hours later, no urease activity could any more be detected.

Discussion

The unpurified exocellular protease of fermentorcultured Streptomyces griseus is at least equipotent with the pancreatic protease official in the pharmacopoeia. The optimum pH for casein digestion lies in the alkaline range. Apart from casein, the enzyme acts on soy protein, fibrin and peptone, too. Under the experimental conditions employed, casein breakdown by the enzyme took place as a monomolecular reaction. At certain enzyme concentrations, digestion of casein was directly proportional to the amount of enzyme present.

As regards the question into which of the 3 groups of proteases [4] the enzyme belongs, it appears that it should be included in the group to which trypsin and pepsin also belong. In support of this view is the fact that no dialyzable co-enzyme could be demonstrated. H_2S, cyanide or cysteine failed to activate the enzyme. These agents, as well as ferrous and manganese ions and serum were either inactive or inhibited the action of Streptomyces griseus protease.

For the presence of a protease in Streptomyces griseus I have found the following data in the pertaining literature. In his book on Actinomycetes, WAKSMAN [5] published a table from a work by LIESKKE and JENSEN (1930), which shows that cultivation on 2 per cent gelatine results in a considerable increase in the amount of amino N titratable with formol. Moreover R. RICHOU et al. [6] in 1949 a proteolytic enzyme demonstrated in unpurified streptomycin preparations.

Our investigations have shown that in the mycelium of Streptomyces griseus there is also active urease. Since mycelium extracts from cultures not containing exogenous urea did also possess urease activity, the enzyme is not produced by adaptative formation. Thus, it could be concluded that the urease in question has a physiological role in the nitrogen metabolism of the strains tested when grown on the medium specified.

Since, according to SUMNER, urease is highly specific, it can be assumed that in the metabolism of Streptomyces griseus urea is also formed. Whether this results from the breakdown of arginine, as common in animals or micro-organisms, or arises in the course of some other metabolic process of Streptomyces griseus, is not known — it is possible that urea is produced from guanidine on the action of guanidase.

Summary

1. In fermentor cultures of Streptomyces griseus in soy bean meal medium a potent exocellular protease is produced within the first 2 days of fermentation.

2. The crude enzymic powder precipitable from the fermentation juice with acetone or with methanol breaks down casein, soy protein, fibrin and peptone. Its action on casein is not weaker than that of pancreatin.

3. The crude enzymic powder has a definite milk-coagulating effect.

4. The optimum pH for casein digestion is around 8,24.

5. Dissolved in distilled water or in a buffered solution of optimum pH, the enzyme is inactivated at 60° C in 30 minutes.

6. The rate of casein breakdown, as well as the relation between the effect on the same substrate and the concentration of the enzyme have been investigated.

7. Dialysis did not decrease the casein digesting potency of the enzyme.

8. H_2S, HCN, or cysteine did not activate the effect of the enzyme on casein. These agents, as well as ferrous sulphate, manganese sulphate and cat serum exerted mainly an inhibitory effect on the enzyme.

9. Urea could not be demonstrated in the myceliumfree centrifugate of the fermentation fluid obtained from fermentor cultures of Streptomyces griseus.

10. Different strains of Streptomyces griseus possess an urease activity when grown in CHRISTENSEN's medium.

11. Mycelial extracts prepared from Streptomyces griseus grown in fermentors in soy bean meal and glucose media form ammonia from urea. The urease activity of mycelial extracts grown in media not containing exogenous urea indicates that urea is produced in the course of some metabolism process of Streptomyces griseus.

Acknowledgement

I am greatly indebted to *Dr. I. Lovrekovich* for making available the fermentation material and for his help in the bacteriological experiments, to *Dr. D. Bagdy* for guidance in the fibrin experiments and to *G. Kálmán* for his devoted and conscientious technical assistance.

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ОБ ЭНЗИМАХ ПРОТЕАЗЫ И УРЕАЗЫ ГРИБКА ACTINOMYCES GLOBISPORUS STREPTOMYCINI (STREPTOMYCES GRISEUS)

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Резюме

Лишенная мицелия ферментационная жидкость микроорганизма, выращенного в ферментаторах на среде из соевой муки, кукурузной гущи и глюкозы, уже в 24 48 часу ферментации выявляет сильное расщепляющее действие на белок сои. Неочищенный сухой порошок энзима, осажденный ацетоном или метанолом, обладает по крайней мере такой же сильной способностью переваривания казеина, как и фармакопейный панкреатин. Кроме казеина и белка сои протеаза расщепляет также фибрин и пептон. Порошок энзима имеет способность свертывания молока. Молоко свернулось при 40 C° от концентрации энзима в 0,5 мг/мл. Оптимальное pH действия казеина в буфере из гликоколя и NaOH колебалось между 7,9 8,4. Порошок энзима в количестве 4 мг расщеплял 5 мл 4%-го раствора казеина при 39 C° в течение первых 90 минут воздействия соответственно мономолекулярной реакции. В случае низких концентраций энзима расщепление казеина прямо пропорционально концентрации энзима. Активность энзима, диализированного в целло ановой гильзе против дестиллированной или проточной воды в течение 24 42 часов – по сравнению с недиализированным контролем снизилась только на 2 4%, так что, по всей вероятности, энзим не содержит диализирующегося коэнзима. HCN, H_2S, цистеин не активизировали казеинорасщепляющего действия энзима. Эти вещества, а также сернокислое железо, сульфат марганца и кошачья сыворотка только тормозили активность энзима.

При выращивании Streptomyces griseus в ферментаторах, в центрифугатах ферментационных жидкостей различного возраста, не содержащих мицеля, мочевины не обнаружено.

Разные штаммы Streptomyces griseus при выращивании на питательной среде Христенсена показали положительную активность уреазы. Экстраты мицелия Streptomyces griseus, выращенные в ферментаторах на питательной зреде из соевой муки и глюкозы, расщепляют мочевину на аммоний.

Активность уреази в экстрактах мицелия, выращенных на питательных средах не содержащих экзогенной мочевины, показывает, что микроорганизм в процессе обмена веществ вырабатывает мочевину.

IMMUNOBIOLOGICAL STUDIES ON ENDOTOXINS

I

INVESTIGATIONS INTO THE ACTIVE ANTIENDOTOXIC PROTECTION IN MICE*

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(Received, February 25, 1955)

In infectious diseases due to pathogenic intestinal bacteria *(S. typhi, S. paratyphi, Sh. dysenteriae)* endotoxic effects are responsible for many of the symptoms, it is therefore of importance to determine to what measure the organism is protected against bacterial endotoxins following actual disease or after vaccination. According to data in the literature (BOIVIN and MESROBEANU [1], GRASSET et al. [2, 3], RAUSS and KÉTYI [4]), only very slight active antiendotoxic protection can be induced in animals. RAUSS [5] found that mice inoculated with a vaccine containing *Sh. flexneri* endotoxin showed a high degree of protection against bacteria, but succumbed to homologus endotoxin in numbers equal with the controls. BOIVIN and MESROBEANU [6], DUBOS [7], RAUSS and KÉTYI [4] suggest that in typhoid fever and in dystentery antiendotoxic protection is of inferior significance, as compared to antibacterial immunity.

In the present paper, studies concerned with the problem of active protection against endotoxins are described.

Materials and methods

Endotoxins. A 20-hour agar culture of strain Ty-O 901 was washed with distilled water and treated with trichlor acetic acid according to BOIVIN and MESROBEANU [8]. After allowing it to stand overnight in a refrigerator, the material was centrifuged, the pH of the supernatant was adjusted to 7,0. After dialysis the material was dried in vacuum, at room temperature. The LD50 of the crude endotoxin powder so obtained was 1,2 mg intraperitoneally. Crude endotoxins of *S. typhimurium* and of *V. cholerae* were prepared similarly. The *Sh. dysenteriae* endotoxin was obtained from I. HOLLÓS. A part of the trichloroacetic acid extract of *S. typhi* organisms was purified by the method of DARVAS and UJHELYI [9]. The toxicity of the endotoxin powder obtained by serial alcoholic precipitation in cold was about one-tenth of the toxicity of the crude powder, the LD50 being 10,0 mg intraperitoneally.

Exotoxins. The *Sh. dysenteriae* exotoxin used was obtained from I. HOLLÓS and the *B. perfringens alpha* and the *Cl. tetani* toxins from L. ERDŐS, to whom we extend our thanks.

Typhoid vaccines. A 20-hour agar culture of strain Ty_2 was washed with saline and the bacterial suspension was divided into several parts. One part was allowed to stand at 60° C for 2 hours, two parts were treated with 0,5 per cent formaldehyde and 0,5 per cent phenol, respectively, and maintained at 37° C for 6 days. A further part was treated with alcohol (final concentration: 50,0 per cent), preserved with 25 per cent alcohol, while another part was

* On the basis of a lecture delivered at the September (1953) Meeting of the Hungarian Microbiological Society.

treated with Merfen (final concentration : 0,1 per cent), to kill off organisms. Germ count of the vaccines was adjusted to 1000 million/ml.

In toxicity tests, fawn-coloured mice ranging in weight from 14 to 16 g were inoculated with 0,5 ml of endotoxin intraperitoneally or intravenously.

Immunization. Fawn-coloured mice ranging in weight from 14 to 16 g were inoculated with 0,5 ml of vaccine or with 0,6 mg of crude *S. typhi* endotoxin, subcutaneously. One week later both the immunized and untreated mice were inoculated with 10 to 100 and 200 to 1,800 million germs of strain Ty_2, respectively, or with 1,2 mg of the crude, or 10 to 20 mg of the purified *S. typhi* endotoxin, by the intraperitoneal route. The volume of the single inocula was invariably 0,5 ml.

Results

In the first part of the work mice were immunized subcutaneously with 0,6 mg of crude *S. typhi* endotoxin, or with 0,5 ml of one of the different typhoid vaccines. One week later one group of mice was given intraperitoneally 200 to 1800 million germs of strain Ty_2, while the other group was treated with 1 to 2 LD50 of *S. typhi* endotoxin, also intraperitoneally. Thus, what we examined was the degree of protection against living bacteria and endotoxin, respectively, that the animals had developed following immunization. The *S. typhi* endotoxin produced a considerable antibacterial immunity, but, under the experimental conditions specified, it has failed to produce protection to endotoxin (Table I). The results obtained were practically the same whether vaccines

Table I

Antibacterial and antiendotoxic protection in mice immunized with S. typhi endotoxin

(0,6 mg of crude endotoxin subcutaneously, followed by infection or intoxication 7 days later)

Immunized mice			Control mice			Immunized mice		Control mice	
Infective dose (million germs)						Toxic dose (mg of crude endotoxin)			
200	600	1800	10	30	100	1,2	2,4	1,2	2,4
*10/12	4/12	1/12	9/12	6/12	2/12	4/10	0/10	5/10	0/10

* Numerator = Number of survivors ; Denominator = Number of mice infected or intoxicated.

treated by heat, formaldehyde, phenol, alcohol or Merfen were tested. A single subcutaneous vaccination produced a high level of antibacterial protection but did not protect the mice from the effects of either the crude of the purified *S. typhi* endotoxin. Thus, our results agree with those of *Rauss* [5] obtained with *Sh. flexneri* endotoxin.

In the above experiments, active immunization against endotoxin was examined after administering a single dose in the range of, or more than, the

lethal dose. The question arose whether the lethal amount of endotoxin should not be introduced in divided doses over a period of several days, this probably being a better approach to natural conditions, since in diseases caused by intestinal bacteria it never occurs that the lethal dose of endotoxin should enter the organism within a short period of time. The endotoxin exerts its effect in a prolonged, gradual manner, as it is released from disintegrating intestinal bacteria. It was thought that immunisation might induce some degree of antiendotoxic protection, which could perhaps be demonstrated by giving the endotoxin in divided doses, but which may be masked or washed off if a single lethal dose of endotoxin is administered.

The first step was to find the adequate dosage of endotoxin for non-immunized mice. It was found that mice which had received a small dose of *S. typhi* endotoxin intraperitoneally or intravenously were protected against several times the formerly lethal dose of endotoxin as early as 24 hours after inoculation. Illustrative of this fact is the following series. Each mouse in a group of 110 was given 0,3 mg of crude *S. typhi* endotoxin intravenously. 21 mice died by next day. The survivors were grouped again and inoculated with 2 to 6 LD50 of *S. typhi* endotoxin 1, 4, 8, 16, 24 hours, 2, 4 and 10 days, respectively, following the initial inoculation (Table II.).

Table II

Repeated inoculation of mice with S. typhi endotoxin

(First inoculum, 0,3 mg of crude *S. typhi* endotoxin, intravenously, second inoculum, 2 to 6 LD50 crude *S. typhi* endotoxin, intravenously)

Interval between the two inoculations												
1 hour	4 hours	8 hours	16 hours		24 hours		2 days			4 days		10 days
2 LD50	2 LD50	2 LD50	2 LD50	3 LD50	2 LD50	6 LD50	2 LD50	3 LD50	4 LD50	2 LD50	3 LD50	2 LD50
*0/8	0/8	0/8	3/8	2/8	8/8	3/6	4/5	4/5	2/5	3/5	1/5	0/5

* Numerator = Number of survivors. Denominator = Number of mice inoculated with endotoxin.

The data in Table II. show that the antiendotoxic protection produced by a small dose of *S. typhi* endotoxin develops rapidly, reaching its maximum within 24 hours. Subsequently it declines quickly and is practically absent by the time antibodies make their appearance.

Protection against the endotoxin of *S. typhi* could be produced not only by intravenous, but also by intraperitoneal administration of the first inoculum,

but in the latter case protection developed less rapidly than after intravenous application.

Subcutaneous administration of *S. typhi* endotoxin produced only slight antiendotoxic protection even if doses as high as 3 or 8 times the usual pretreatment-dose had been applied.

In order to determine the degree of specificity of the protection resulting from the above kind of treatment, 24 hours after intravenous administration of *S. typhi* endotoxin the test animals were given *S. typhimurium*, V. cholerae or *Sh. dysenteriae* endotoxin by the intravenous route, or *Sh. dysenteriae* exotoxin intraperitoneally, or *B. perfringens* alpha-toxin intravenously, or *Cl. tetani* toxin subcutaneously. (Tables III—VIII) The results in the Tables

Table III

Effect of S. typhimurium endotoxin in control and in S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intravenously, followed by *S. typhimurium* endotoxin, intraperitoneally, 24 hours later)

Pretreated animals				Controls			
Dose of S. typhimurium endotoxin in mg							
1,0	2,0	4,0	8,0	1,0	2,0	4,0	8,0
*8/8	7/8	3/8	0/8	5/6	0/6	0/6	0/6

* Numerator = Number of survivors. Denominator = Number of mice treated with S. typhimurium endotoxin.

Table IV

Effect of V. cholerae endotoxin in control and in S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intravenously, followed by *V. cholerae* endotoxin, intraperitoneally, 24 hours later)

Pretreated animals				Controls			
Dose of V. cholerae endotoxin in mg							
0,5	1,0	2,0	4,0	0,5	1,0	2,0	4,0
*8/8	7/8	5/8	0/8	6/6	0/6	0/6	0/6

* Numerator = Number of survivors. Denominator = Number of mice treated with V. cholerae endotoxin.

Table V

Effect of Sh. dysenteriae endotoxin in control and in S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intravenously, followed by *Sh. dysenteriae* endotoxin, intraperitoneally, 24 hours later)

Pretreated animals				Controls			
Dose of Sh. dysenteriae endotoxin in mg.							
0,3	0,5	0,7	1,0	0,3	0,5	0,7	1,0
*8/8	8/8	7/8	5/8	2/4	0/4	0/4	0/4

* Numerator = Number of survivors. Denominator = Number of mice treated with Sh. dysenteriae endotoxin.

Table VI

Effect of Sh. dysenteriae exotoxin in control and in S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intravenously, followed by *Sh. dysenteriae* exotoxin, intraperitoneally, 24 hours later)

Pretreated animals				Controls			
Dose of Sh. dysenteriae exotoxin in mg.							
1,0	3,0	5,0	7,0	1,0	3,0	5,0	7,0
*8/8	7/8	1/8	0/8	8/8	8/8	3/8	0/8

* Numerator = Number of survivors. Denominator = number of mice treated with Sh. dysenteriae exotoxin.

Table VII

Effect of B. perfringens alpha toxin in control and S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intravenously, followed by a mixture of *B. perfringens* alpha toxin—antitoxin, intravenously, 24 hours later)

Pretreated animals				Controls			
Dose of B. perfringens alpha toxin in ml.							
0,15	0,20	0,25	0,30	0,15	0,20	0,25	0,30
*4/4	4/4	2/4	0/4	4/4	4/4	2/4	0/4

* Numerator = Number of survivors. Denominator = number of mice treated with B. perfringens alpha toxin.

Table VIII

Effect of Cl. tetani toxin in control and S. typhi endotoxin-pretreated mice
(0,3 mg of *S. typhi* endotoxin, intraperitoneally, followed by a mixture of *Cl. tetani* toxin—antitoxin, subcutaneously, 24 hours later)

Pretreated animals				Controls			
Dose of Cl. tetani toxin in ml							
0,10	0,15	0,20	0,25	0,10	0,15	0,20	0,25
*0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Following inoculation with *Cl. tetani* toxin, death occurred after							
5 days	3 days	2 days	2 days	5 days	3 days	2 days	2 days

* Numerator = Number of survivors. Denominator = Number of mice treated with Cl. tetani toxin.

reveal that intravenously administered *Sh. typhi* endotoxin protects not only against the homologous endotoxin but also against *S. typhimurium*, *V. cholerae* and *Sh. dysenteriae* endotoxins, while it does not lend protection against *Sh. dysenteriae* exotoxin, *B. perfringens* alpha-totin and *Cl. tetani* toxin.

Discussion

In studies on the protection against endotoxins, the central problem is in most cases to clarify, what protection is afforded by antibodies in the immunized organism against the toxic effects of endotoxins. For this reason, in active protection tests mice immunized subcutaneously or intraperitoneally, or rabbits immunized intraperitoneally or intravenously are given homologous endotoxin mostly at the peak of antibody production. Under such experimental conditions rabbits usually develop antiendotoxic protection, while mice do not. The explanation of this phenomenon will be dealt with in another paper [11].

In agreement with data in the literature it was found that, after a single subcutaneus inoculation of one of various typhoid vaccines prepared by different methods, or of *S. typhi* endotoxin mice developed only antibacterial, but no antiendotoxic immunity. On the other hand, mice inoculated intravenously or intraperitoneally with a small dose of *S. typhi* endotoxin developed resistance to several times the lethal dose of endotoxin in 16 to 24 hours. This rapidly developing resistance cannot be ascribed to antibodies since it protects against homologous and heterologous endotoxins alike.

The above experimental results are in connection with those reported by MORGAN et al.(12—14) who have found that, both in man and in the rabbit, fever and other toxic symptoms due to endotoxin can be diminished by previous and repeated endotoxin administration. This tolerance is not based on the presence of antibodies, it being non-specific in nature and extending to endotoxins which, although related chemically and toxicologically, differ in serological specificity. The human organism may develop a high degree of resistance to the pyrogenic effect of endotoxins. So, for instance, after intravenously administering typhoid vaccine 9 times, the dose of antigen required for producing fever comparable in duration and intensity to that induced by the first inoculation, was 7000 times the first dose [14]. MORGAN has not studied whether the antipyretic tolerance affords at the same time protection against the lethal effect of endotoxin. It is known that different degrees of tolerance may develop to different effects of certain biologically active agents [15]. For example, dogs that had acquired resistance to the respiratory rate decreasing effect of morphine, or rats with increased tolerance to the anaesthetic effect of that drug, are equally or more susceptible to lethal doses of morphine than untreated controls [16]. We have succeeded in demonstrating that mice can be induced to develop resistance to several times the lethal dose of endotoxins. The results obtained in similar studies by RÉDEY [17] are practically in agreement with ours. Tolerance to the pyrogenic action, however, does not indicate necessarily resistance to the lethal effect of endotoxin [11]. Resistance can be induced against the tumour-necrosing [18], white cell migration inhibitory [19], and the SHWARTZMAN phenomenon provoking effects [20] of endotoxins.

In the light of the above it is obvious that the organism can be induced to develop resistance to various endotoxic effects. However, the mechanism of that protection still awaits elucidation. According to MORGAN [12] and BEESON [21], it is the reticuloendothelial system which eliminates pyrogenic agents from the circulation of animals with acquired resistance. The resistance to the pyrogenic effect of endotoxins can be suspended by blocking the reticuloendothelial system [21]. The fact that the mode of endotoxic action is also unknown adds to the difficulties in studies of the subject. (A competent survey of the theories concerning the mode of action of endotoxins has been published by BURROWS [22]). The view according to which endotoxins would exert their deleterious action through the mediation of the nervous system [23] merits considerable attention. ADO [24] showed that introduction of certain antigens into the organism may cause, directly or reflectorily, profound changes in systemic responsiveness to the given antigen or to subsequent exposure to other stimuli. GORSHOLEVA and HOZAK [25] reported that staphylococcal toxin produced diffuse inhibition in the cerebral cortex of the rat. These and numerous similar data appear to justify the view that in studies on resistance to endotoxins attention should be devoted to the laws of non-specific immunity, too.

Reports concerned with non-specific immunity have been published since long. As early as in the nineties, PFEIFFER and ISSAEFF [26] had shown that by non-specific treatment (for example by administering coli, typhoid, or pyocyaneus organisms intraperitoneally) guinea pigs can be induced to develop immunity to virulent cholera vibrios within 24 hours. ISSAEFF [27] had observed comparably good results after intraperitoneal injection of broth, nucleic acid, tuberculin, urine, and other materials. Later, numerous similar observations were reported in the literature. According to OERSKOV and KAUFFMANN [28], white mice inoculated with E. coli vaccine develop immunity to typhoid or dysentery infection within 24 hours. OERSKOV and KAUFFMANN have suggested the term «promunity» to denote an alteration in responsiveness induced by non-specific treatment. Promunicy, studied in detail by OERSKOV [29] and PHILIPSON [30], depressive immunity (MORGENROTH *et al.* [31], SCHNITZER and KÜHLEWEIN [32]), as well as «premunition» (SERGENT [33]) represent single phenomena of non-specific immunity (BRANDIS [34]). The material accumulated on the subject is enormous. A proper interpretation of the not infrequently conflicting experimental results and an adequate evaluation of the various theories advanced can be achieved only on the basis of a uniform biological conception [35, 36, 37].

Summary

1. Mice subcutaneously inoculated with typhoid vaccines prepared by different methods or with *S. typhi* endotoxin develop a high degree of antibacterial, but no endotoxic, immunity.

2. Mice given single small doses of *S. typhi* endotoxin intravenously or intraperitoneally develop resistance to several times the lethal dose of the homologous endotoxin. Protection was demonstrable as early as 16 hours after inoculation, reaching its peak at 24 hours. Subsequently a sharp decline occurred and resistance was practically non-existent by the time antibodies appeared in the blood.

3. By intravenous administration of *S. typhi* endotoxin mice can be rendered resistant not only to homologous endotoxin, but also to those of *S. typhimurium*, *V. cholerae* and *Sh. dysenteriae*. But they remain unprotected against *Sh. dysenteriae* exotoxin, *B. perfringens* alpha toxin and *Cl. tetani* toxin.

4. The role of non-specific immunity in active antiendotoxic protection has been discussed.

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ИММУНОБИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ЭНДОТОКСИНОВ

I. Испытание активного антиэндотоксического иммунитета в опытах на мышах

Ш. БОЖОКИ

Резюме

Симптомы—инфекционных заболеваний, вызываемых патогенными кишечными бактериями (тиф, паратиф, дизентерия), развиваются главным образом под действием эндотоксинов соответствующих бактерий. Итак, является важным исследовать, насколько можно сделать организм иммунным к токсическому действию эндотоксинов. Автор иммунизировал мышей подкожно вакцинами, изготовленными различными способами (вакцины убитые нагреванием, формалином, карболовой кислотой, алкоголем, мерфеном), и тифозным эндотоксином. Спустя 7 дней после иммунизации одну часть мышей заражал внутрибрюшинно соответствующими дозами живых бактерий Ty_2, а другую часть внутрибрюшинно отравлял тифозным эндотоксином. У иммунизированных мышей выявлялся весьма выраженный антибактериальный иммунитет, в то время как от тифозного эндотоксина они погибали так же как и неиммунизированные контрольные животные. Следовательно путем подкожного введения тифозных вакцин или тифозного эндотоксина мыши не приобретали иммунитета к тифозному эндотоксину.

Согласно дальнейших исследований автора однако имеется возможность выработки значительного антибактериального иммунитета посредством внутривенного введения малой дозы тифозного эндотоксина. Иммунитет этот обнаруживается уже спустя 16 часов после предварительной внутривенной обработки и достигает максимума через

24 часа. В это время мыши не погибают даже от 2–6 LD_{50} тифозного эндотоксина. Степень антиэндотоксического иммунитета затем чрезвычайно быстро понижается и во время появления антител может считаться прекращенным. Иммунитет к тифозному эндотоксину может быть выработан также и путем внутрибрюшинного введения тифозного эндотоксина, но возникает медленнее, чем в случае внутривенной прививки. Иммунитет, вырабатываемый у мышей путем внутривенного введения тифозного эндотоксина, является эффективным не только по отношению к тифозному эндотоксину, но и к эндотоксинам **S. typhimurium, V. cholerae** и **Sh. dysenteriae,** однако не действителен против экзотоксина **Sh. dysenteriae, α**-токсина **B. perfringens** и токсина **Cl. tetani.**

Согласно вышеприведенному антиэндотоксический иммунитет, вызванный у мышей путем внутривенного или внутрибрюшинного введения малой дозы тифозного эндотоксина, не обоснован наличием антител, а является последствием неспецифической резистентности, развивающейся по отношению к эндотоксинам.

IMMUNOBIOLOGICAL STUDIES ON ENDOTOXINS

II

INVESTIGATIONS INTO THE ACTIVE ANTIENDOTOXIC PROTECTION IN RABBITS*

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It is known that rabbits immunized with endotoxins acquire protection against several the lethal dose of the homologous endotoxin. It is generally believed that this protection is so low because the antibodies formed against endotoxins have but a limited neutralizing capacity [1—9,13.]. In an earlier study [10], however, it was shown by us that mice immunized subcutaneously with *S. typhi* endotoxin or with typhoid vaccines are not protected against the homologous endotoxin at the time antibodies appear in the blood. It seemed, therefore that active antiendotoxic protection tests yield different results in mice and in rabbits. The explanation of this discrepancy has been the purpose of the studies to be described.

Materials and methods

Endotoxins. *S. typhi* and *S. typhimurium* endotoxins, extracted with trichloroacetic acid [10] were used, as well as *Sh. flexneri*-5 endotoxin, obtained from I. Hollós.

Immunization. Rabbits ranging in weight from 3000 to 4000 g were inoculated at 3-day intervals with increasing doses of a Ty-O 901 bacterial suspension intravenously. The bacterial suspension had been boiled for 1 hour before use and its germ count was 5000 million/ml; the single doses were 0,5, 1,0, 1,5, 2,0, 3,0 and 3,0 ml. Three days after the sixth inoculation the animals were administered 2 to 7 lethal doses of *S. typhi*, *S. typhimurium* or *Sh. flexneri* endotoxin by the intravenous route. The surviving animals were given further doses of endotoxin within the following few days. At the onset, and at intervals during the experiment the rabbits were tested for Ty-O agglutinin titre.

Pyrogen Tests. Rabbits ranging in weight from 3000 to 4000 g were given, at 3-day intervals and by the intravenous route, 2500, 5000, 10 000 and 10 000 million germs of the acetone-treated and dried strain Ty-O 901. Prior to, and 2, 4 and 6 hours following inoculation the rectal temperature of the animals was taken. Three days after completion of the pyrogen tests the rabbits were given *S. typhi* endotoxin intravenously.

Results

First it was studied whether there exists a relationship between the level of Ty-O agglutinins in the untreated rabbit and its resistance to *S. typhi* endotoxin (Table I) According to the data in Table I, there is no such relationship.

* On the basis of a lecture delivered at the September (1954) Meeting of the Hungarian Microbiological Society.

The MLD of the endotoxin used is estimated to be in the range of 2,0 mg/kg. Also, it can be read from Table I that in most cases even the normal antibodies present in the untreated animal have sufficed serologically to neutralize the test doses of endotoxin, since it occurred only in two animals (rabbits No. 5 and No. 9) that in the samples of blood taken 2 hours after administering the endotoxin the antibody level was lower than the initial titre. On the other hand, following endotoxin administration the agglutinin titres remained unchanged (rabbits No. 12, 11, 16, 3 and 6), or were increased (rabbits No. 4, 1, 7).

Table I

Effect of intravenous S. typhi endotoxin on untreated rabbits

S. typhi endotoxin mg/kg	Rabbit No.	Day after intoxication			Ty-O agglutinin titre	
		1	2	40	2 hrs before intoxication	2 hrs after intoxication
1,25	2	*+			1 : 8	—
	12	0	0	0	1 : 8	1 : 4
	4	0	0	0	1 : 16	1 : 64
	5	0	0	0	1 : 128	1 : 8
1,5	1	0	+		1 : 32	1 : 128
	11	+			1 : 512	1 : 512
	9	0	0	0	1 : 128	1 : 32
2,0	16	+			1 : 64	1 : 128
	10	+			1 : 128	—
2,5	3	+			1 : 512	1 : 512
3,2	13	+			1 : 8	—
3,7	7	0	0	0	1 : 32	1 : 512
4,0	6	+			1 : 512	1 : 512
6,2	8	+			1 : 16	—

* 0 = alive + = dead.

In a further series rabbits were immunized with increasing doses of *S. typhi* killed by exposure to heat. (The single intravenous doses of the bacterial suspension containing 5000 million germs per ml were 0,5, 1,0, 1,5, 2,0, 3,0, and 3,0 ml.) Three days after the sixth inoculation the animals were injected with 6,0 to 14,0 mg (i. e. 3 to 7 MLD) of *S. typhi* endotoxin intravenously (Table II).

Table II

Effect of intravenous S. typhi endotoxin on rabbits immunized intravenously with killed S. typhi organisms

S. typhi endotoxin mg/kg	Rabbit No.	Day after intoxication			Ty—O agglutinin titre	
					2 hrs before	2 hrs after
		1	2	40	intoxication	
6,0	26	*+			1 : 102 400	1 : 102 400
7,0	24	0	0	0	1 : 102 400	1 : 102 400
7,5	25	0	0	0	1 : 25 600	1 : 51 200
8,3	20	0	0	0	1 : 102 400	1 : 102 400
9,0	21	+			1 : 12 800	1 : 25 600
12,0	23	0	0	0	1 : 102 400	1 : 51 200
14,0	22	+			1 : 51 200	1 : 51 200

* 0 = alive. + = dead.

It is shown in Table II that rabbits immunized with killed *S. typhi* have acquired protection against only a few lethal doses of the homologous endotoxin, in spite of the fact that the blood antibody level was very high. Considering that *in vitro* as little as 1,0 ml of an 1 : 25.000-titre homologous rabbit antiserum is more than enough to bring about serological neutralization i. e. precipitation of dose of *S. typhi* endotoxin lethal for rabbits [11], the question arises whether antibodies at all neutralize the toxic effect of endotoxin? Is it not possible that the immunized rabbits owe their protection against a few lethal doses of endotoxin beside the presence of antibodies to other factors?

In the above rabbit experiments, immunization was performed by the intravenous route. In our earlier studies [10] it had been shown that mice can also be made resistant to endotoxins by intravenous endotoxin treatment. This protection is non-specific in nature and is not based on the presence of antibodies. In order to establish whether or not the antiendotoxic protection in rabbits is, at least in part, due to development of non-specific resistance, another group of rabbits had been immunized with killed typhoid organisms by the above described method. On the 3rd day after the 6th inoculation, however, the animals were given intravenously increasing doses of *S. typhimurium* or of *Sh. flexneri* endotoxin, instead of the *S. typhi* endotoxin used in the above series. (Tables III & IV)

According to the data in Table III and in Table IV, rabbits immunized intravenously with typhoid organisms were protected against the endotoxin of *S. typhimurium*, which is antigenically related to *S. typhi*, and against that of *Sh. flexneri*, which is serologically indifferent.

Table III

Effect of intravenous S. typhimurium endotoxin on rabbits immunized intravenously with killed S. typhi organisms and on untreated controls

Untreated animals					Immunized animals					
S. typhimurium endotoxin mg/kg	Rabbit No.	Day after intoxication			S. typhimurium endotoxin		Rabbit No.	Day after intoxication		
		1	2	40	mg/kg	MLD/kg		1	2	40
10,0	118	*+			6,0	6	102	*+		
3,0	117	+					123	+		
1,0	122	0	+		4,0	4	129	0	+	
0,5	137	0	0	0			139	0	0	0
					3,0	3	116	0	0	0
					2,0	2	136	0	0	0

* 0 = alive. + = dead.

Table IV

Effect of intravenous Sh flexneri endotoxin on rabbits immunized intravenously with killed S. typhi organisms and on untreated controls

Untreated animals					Immunized animals					
Sh. flexneri endotoxin mg/kg	Rabbit No.	Day after intoxication			Sh. flexneri endotoxin		Rabbit No.	Day after intoxication		
		1	2	40	mg/kg	MLD/kg		1	2	40
6,0	115	*+			10,0	5	112	*+		
4,0	119	+			8,0	4	110	0	+	
2,0	130	+			6,0	3	126	0	0	0
							106	0	0	0
1,0	134	0	0	0	4,0	2	111	0	0	0
							124	0	0	0

* 0 = alive. + = dead.

The 3rd day after the first intravenous inoculation of *S. typhimurium* or *Sh. flexneri* endotoxin a number of rabbits immunized with killed typhoid bacteria was given 2 to 4 MLD of the corresponding endotoxin intravenously. The animals survived this repeated intoxication.

The observations appear to indicate that the protection against endotoxins, produced by intravenous immunization with killed typhoid bacteria, is, at least in part, due to the development of a non-specific resistance to endotoxins. A certain degree of endotoxin-neutralization by specific antibodies cannot, however, be left unconsidered. This problem will be dealt with later.

It has been found by MORGAN [12] that rabbits may be made resistant to the pyrogen action of endotoxins. In the series described below we have examined in comparative tests the degree of resistance to the pyrogen action of *S. typhi* endotoxin and that of protection against lethal endotoxic intoxication. Rabbits ranging in weight from 3000 to 4000 g were inoculated at 3-day intervals with 2500, 5000, 10 000, 10 000 and 10 000 million acetone- killed typhoid bacteria intravenously. Prior to and 2, 4 and 6 hours following inoculation the rectal temperature was taken. The animals developed various degrees of resistance to the pyrogen action of typhoid endotoxin. On the basis of the intensity of febrile reaction the animals were divided into 3 groups, with mild, medium and marked resistance, respectively. Three days after completion of the tests, each animal was given 6,0 mg/kg (i. e. 3 MLD/kg) of *S. typhi* endotoxin intravenously. The survivors were given next day another dose of 6,0 mg/kg of endotoxin. (Table V.) The data in Table V. reveal that resistance to the pyrogenic and that to the lethal effect of *S. typhi* endotoxin do not develop parallelly, as a rule. Although all the animals exhibiting a marked resistance

Table V

Relationship between Ty-O agglutinin titre, acquired resistance to the pyrogenic action of S. typhi endotoxin and degree of antiendotoxic protection, as determined in rabbits intravenously immunized with killed S. typhi organisms

Rabbit No.	Degree of acquired resistance to the pyrogen action of S. typhi endotoxin	Level of Ty-O agglutinin 2 hours before first intoxication	1st day after first injection of endotoxin	Days after 2nd dose of endotoxin		
				1	2	40
72	mild	1 : 40 960	*0	+		
68		1 : 20 480	+			
60		1 : 81 920	+			
71		1 : 20 480	0	0	+	
65		1 : 81 920	0	0	0	0
62	medium	1 : 40 960	+			
61		1 : 40 960	0	0	0	0
64		1 : 20 480	+			
66		1 : 40 960	0	0	+	
63		1 : 40 960	0	+		
69	marked	1 : 20 480	0	+		
70		1 : 40 960	0	+		
67		1 : 40 960	0	0	0	0

* 0 = alive. + = dead.

to pyrogen action had survived the first 6,0 mg/kg dose of endotoxin, only one animal in each of the three groups has survived the second intoxication (i. e. a total of 12.0 mg/kg of endotoxin). No relationship could be demonstrated between antibody titre and resistance to pyrogen or to lethal endotoxin action.

Discussion

Mice immunized in the usual manner with typhoid vaccines or with *S. typhi* endotoxin show no protection against the homologous endotoxin at the time antibodies appear in the blood, while rabbits immunized with *S. typhi* endotoxin do. In our opinion, this is due to the fact that the common method of rabbit immunization produces antiendotoxic resistance, while the common method of mouse immunization fails to do this. When immunizing mice by the intraperitoneal or intravenous routes intoxication with endotoxin is usually carried out 7 days after the immunizing dose had been administered, at a time when the antiendotoxic resistance that had developed as a result of intraperitoneal or intravenous pretreatment, has already disappeared [10]. With rabbits it is also common practice to give the intoxicating dose several days after the last immunizing inoculation. At that time, however, the rabbits exhibit a marked protection against endotoxins, as the antiendotoxic resistance produced by the four or more intravenous or intraperitoneal inoculations persists for as long as several weeks. Antiendotoxic resistance of comparable duration can be induced also in mice by giving several intravenous or intraperitoneal inoculations at adequate intervals [14, 15].

The antiendotoxic resistance produced in the rabbit by repeated intravenous inoculations with *S. typhi* endotoxin is not specific in nature, as it applies not only to the homologous endotoxin, but also to the endotoxins of *S. typhimurium* and *Sh. flexneri*. The experimental results, however, reveal that rabbits immunized with typhoid bacteria are better protected against the homologous endotoxin than against the heterologous ones. The following explanation is put forward to explain this phenomenon. The antibodies of the immunized animal precipitate *in vivo* the homologous endotoxin injected. Thus, as a result of the reaction with antibodies, the physico-chemical properties of the endotoxin in solution undergo changes including a diminution of dispersity i. e. a reduction in the size of the active surface. This, in turn, results in a decrease of toxicity. The substantial role of the degree of dispersity in the activity of biologically active substances is well-known.

Thus, along with the development of a non-specific resistance, specific antibodies may also be involved in the development of active antiendotoxic protection. Passive protection tests also indicate that, even if to a small degree, the immune serum is capable of diminishing the effect of homologous endotoxin [1—9]. The degree of neutralization, however, is not proportional to the volume

of serum and cannot be increased above a certain level. According to MORGAN [6], the precipitate formed on adding an excess of homologous immune serum to *S. typhi* endotoxin remains toxic, although less so than an equal amount of endotoxin not treated with immune serum. DARVAS, BORN and ÚJHELYI [16] reported that rabbit sera of a high neutralization titre can be produced against the endotoxin of V. cholerae. On the other hand, RAUSS and KÉTYI [17] were unable to detect endotoxin-neutralizing antibodies in patients convalescing after dysentery due to *Sh. flexneri* or after typhoid fever.

In our opinion, the serologic reaction between endotoxins and specific antibodies takes place by the following mechanism. The antibodies produced on exposure to endotoxins can neutralize homologous endotoxins only in the serological sense of the word. This antigen-antibody union, however, unlike the combination of exotoxins with antitoxins, is by itself insufficient to reduce the toxic activity of endotoxins. The slight decrease in toxicity observable in passive protection tests may be the result of a decrease in the dispersity of the endotoxin, in consequence of the serologic reaction between endotoxin and homologous antibodies, be it either *in vitro* or *in vivo*. This would explain why the degree of endotoxin neutralization by antibodies does not follow stoechiometric proportions (which, in a certain range, are valid for the exotoxin-antitoxin union). It becomes further clear why the toxicity of endotoxin cannot be completely abolished, whatever great the amount of serum added. Finally, in passive protection tests aspecific factors must also be considered, especially if the serum has been injected intraperitoneally or intravenously. Normal rabbit or horse sera given intraperitoneally may namely produce an increase in aspecific resistance as early as 16 to 24 hours following inoculation [15, 18].

What accounts for the fact that the antibodies produced on treatment with endotoxins do not neutralize the toxicity of the latter? In enzymology there are a number of similar phenomena. For example, the antiserum to catalase or to *E. coli*-beta-galactosidase fails to inhibit the action of the corresponding enzyme [19, 20]. Studies on the structure-function relationship of proteins have elucidated that the different functions of biologically active substances are bound to the presence, or intactness, of definite active centres in the molecule [21, 22]. However, the active centres responsible for enzymic activity and antigenicity, respectively, are not necessarily identical. If the group responsible for enzymic activity is not involved in antibody production (either because it is non-polar in nature or because it is located to the centre of the molecule), the antibodies that develop precipitate the enzyme without affecting its activity. Presumably the same applies to *S. typhi* endotoxin [6] and to endotoxins in general.

The chemical structure of endotoxins has been studied by many authors [23—34], but the nature of the active group responsible for toxicity could not yet be determined. The investigations by TAL and GOEBEL [35] merit

particular attention. They show that the toxicity of *Sh. flexneri* endotoxin is not bound to the lipid, polysaccharide or protein fractions ; the carrier of toxicity is a closely not identified substance containing phosphorus, constituting but a small fraction of the entire endotoxin-complex.

On the basis of the above it appears that endotoxins are poisons closely related to somatic antigen. In immunology toxins mean such poisonous animal or plant constituents which induce the organism to produce antibodies. Poisons of non-antigenic nature (such as acetanilide, strychnine, atropine) do not induce antibody production. On the other hand, the organism may develop, on grounds of a mostly unknown mechanism, a resistance to poisons [36].

The antibodies produced on exposure to endotoxins «neutralize» endotoxins only in the serological sense of the word, but do not abolish the toxic action of the latter. On the other hand, the organism may be made highly resistant to endotoxins, as also to poisons. Endotoxins may be therefore ntermediate forms between toxins and poisons.

Summary

1. In untreated rabbits no parallelism exists between the normal level of agglutinin to Ty-O and the resistance to *S. typhi* endotoxin.

2. Rabbits intravenously immunized with killed *S. typhi* become protected against a few lethal doses of *S. typhi* endotoxin.

3. In immunized animals no relationship exists between antiendotoxic protection and the level of O agglutinins.

4. Rabbits immunized intravenously with killed *S. typhi* organisms become resistant to a few lethal doses of *S. typhimurium* and *Sh. flexneri* endotoxins as well.

5. Resistance to the pyrogenic action of *S. typhi* endotoxin does not necessarily indicate the degree of resistance to the lethal action of that endotoxin.

6. The nature of endotoxins and that of the reaction of endotoxins with homologous antibodies has been discussed.

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ИММУНОБИОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ЭНДОТОКСИНОВ

II. Испытание активного антиэндотоксического иммунитета в опытах на кроликах

Ш. БОЖОКИ

Резюме

В первую очередь автор определил, что у неиммунизированных кроликов не существует параллелизма между титром нормального тифозного О-агглютинина и резистентностью к тифозному эндотоксину. Затем иммунизировал кроликов внутривенно повышающейся дозой убитых нагреванием тифозных бактерий, с интервалами по 3 дня. Через 3 дня после 6 прививок внутривенно отравлял животных тифозным эндотоксином в количестве 3—7 MLD. Иммунизированные убитыми тифозными бактериями кролики оказались невосприимчивыми только к нескольким смертельным дозам гомологичного эндотоксина, несмотря на то, что обладали весьма высоким титром агглютининов. Нельзя было определить параллелизма между титром тифозного О-агглютинина и антиэндотоксическим иммунитетом иммунизированных животных. Кролики, иммунизированные убитыми тифозными бактериями, приобретали иммунитет к нескольким летальным дозам эндотоксинов не только тифозных бактерий, но и находящихся с таковыми в антигенной связи бактерий S. typhimurium, а также серологически индиферентных Sh. flexneri. Описанные результаты опытов указывают на то, что антиэндотоксический иммунитет, возникающий у кроликов вследствие внутривенного введения убитых тифозных бактерий, не обосновывается наличием антител, а объясняется развитием неспецифической резистентности к эндотоксину.

Под действием повторных внутривенных прививок эндотоксина кролики становятся резистентными также и к пирогенному действию эндотоксина. Резистентность к пирогенному действию эндотоксинов однако не всегда параллельна степени иммунитета, выявляющегося по отношению к летальной дозе эндотоксина.

По мнению автора серологическая реакция, развивающаяся между эндотоксинами и специфическими антителами, объясняется следующим образом. Антитела, возникающие под действием эндотоксинов, способны «нейтрализовать» эндотоксины только с серологической точки зрения. Это связывание антигена-антител однако само собой не понижает токсического действия эндотоксинов. Незначительное понижение токсичности, наблюдаемое вследствие преципитации эндотоксинов, объясняется понижением дисперсности эндотоксина.

Согласно описанных опытов организм можно сделать в большой степени резистентным к эндотоксинам, как это известно в связи с ядами. Таким образом эндотоксины занимают срединное место между токсинами и ядами.

THE HAEMOLYTIC ACTIVITY OF CHLORAMPHENICOL-RESISTANT SALMONELLA TYPHI

By

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The mechanism of bacterial resistance to antibiotics or chemotherapeutics can establish itself in various ways. EHRLICH [1] already pointed out that the protoplasm of some trypanosoma resistant to arsenic compounds contains considerably less arsenic than that of the sensitive strains. Strains of *Staphylococcus pyogenes*, made *in vitro*, resistant to penicillin were observed to behave similary in environments containing penicillin salt traced with a radioactive isotope [2]. A far greater number of penicillin molecules accumulated in the sensitive cells than in the resistant ones. These observations tend to show that cellular surface and changes in its permeability are of major significance in the development of resistance.

Another way in which bacteria resist antibiotics and/or chemotherapeutics is to activate the production of enzymes capable of breaking down the surrounding bacteriostatic or bactericide substances. This is instanced by the penicillinase enzyme of staphylococci resistant to penicillin *in vivo* [3].

Still another way in which microorganisms protect themselves against inhibitors is to produce an excess of that essential metabolite which is competitively inhibited by the applied chemotherapeutic. For instance, some — though not all — strains of *Neisseria gonorrhoeae* resistant to sulfonamides, produce an increased amount of p-aminobenzoic acid [4]. In addition to these principal forms of mechanism, there is a great variety of others, as yet unelucidated in detail, that can give rise to drug resistance.

Our experiments aimed at obtaining a clearer view of the mechanism by which *S. typhi* become resistant to chloramphenicol *in vitro*. It was found that, in the course of turning resistant, the examined strains acquired haemolytic activity.

Materials and methods

With a view to adapting *S. typhi* strains to chloramphenicol, doses of 0,1 ml of 24-hour broth cultures were inoculated into a series of tubes with broth containing the antibiotic in increasing concentration (1—2—4—8 γ/ml, etc.). After incubation for 48 hours, broth containing a still higher concentration of the antibiotic was inoculated with 0,1 ml of that culture in which growth had still been complete.

Determination of haemolysis. Plating *S. typhi* strains on blood agar, their haemolysing activity was read after incubation for 48 hours at 37° C.

Isolation of the haemolysing agent. Of a broth containing 2 per cent glucose, 125 ml were inoculated with 2 ml of the 24-hour bouillon culture of a resistant *S. typhi* strain. After incubation for 24 hours at 37° C, the culture was centrifuged for half an hour at 3000 r. p. m. The cell-free supernatant containing the haemolysing agent was then used in the investigations.

Measuring haemolytic activity. A geometrically graded series of dilutions of a total volume of 2 ml was prepared with physiological saline from the cell-free supernatant. After adding to each of the tubes 0,5 ml of a 2 per cent suspension of washed sheep erythrocytes, and incubating them for an hour in a water bath at 37° C, the last tube was verified in which the erythrocytes had completely dissolved : the ratio of dilution in that tube served as the titre of the haemolysin

Experimental

Nine strains of *S. typhi* of different phage types were used in the experiments. On growing the sensitive strains in broth cultures containing concentrations of chloramphenicol graded up in the way described above, it was found

Fig. 1. Colonies of chloramphenicol-resistant *S. typhi* on blood agar after 24 hours

that after 4 to 6 weeks even concentrations 50 times as high as those initially tolerated did not stop their propagation. Only differences of degree manifested themselves in the resistance displayed by the *S. typhi* strains of different phage type.

In agreement with SAGEBIEL's [5] findings, no qualitative difference between sensitive and resistant strains was found in regard to their capacity of breaking up sugars and alcohols. A different behaviour was manifested by resistant strains when inoculated on blood agar, for after incubation for 48 hours at 37° C their colonies were surrounded by a beta haemolytic halo from 1 to 2 mm wide (Fig. 1).

The *S. typhi* strains resistant to chloramphenicol have thus revealed a new quality, the capacity to dissolve red blood corpuscles. This quality appeared simultaneously with the strains' turning resistant. The haemolysing capacity grows with growing resistance, and disappears when the resistance weakens or disappears.

The haemolytic agent produced belongs to the group of bacteriolysins. In order to ascertain its qualities and determine its proper place among the other bacteriolysins, the following experiments were made.

First, production on a liquid culture medium was studied. On examining, in the manner described in the section on methods, the cell-free supernatant of the resistant *S. typhi* strain in a 24-hour bouillon culture containing 2 per cent glucose, it was found to possess haemolysing capacity. A 1 : 10 dilution of the supernatant completely dissolved 0,5 ml of a 2 per cent suspension of washed sheep erythrocytes during incubation for 1 hour in a water bath at 37° C. On examining several series of cultures, the haemolytic titre of the supernatant was found, on an average, to be a dilution of 1 : 10.

These experiments have shown that haemolytic activity develops not only on solid nutrient media but it is also demonstrable in the cell-free supernatants of broth cultures containing 2 per cent glucose.

No essential change in the haemolytic titre of the supernatant occurred on using erythrocytes of man, rabbit, guinea pig or even those of mouse, instead of red blood corpuscles of sheep.

Among lytic bacterial enzymes, strepto- and staphylolysins form well definable groups. The two groups differ in the following. *Streptolysins* are oxygen labile, are reversibly inactived by oxidizing agents, their lytic effect is suspended by cholesterol, and they dissolve mouse erythrocytes in a lower titre. Mouse erythrocytes, accordingly, are in some degree resistant to them. Immune serum produced with any of the lysins of this group acts as an inhibitor the lytic ferments of all members of the group (VAN HEYNINGEN, 1950 [6], TODD, 1941 [7], HOWARD et al., 1953 [8]).

Staphylolysins, on the other hand, are oxygen stabile, their effect is increased by reducing agents, cholesterol does not affect them, and lysis is inhibited by staphylo alpha antitoxic serum (HOWARD [8]).

The effect of these substances on the haemolytic agent produced by resistant *S. typhi* is shown in Table I.

A 1 : 10 concentration of the haemolysin in the supernatant dissolves the sheep erythrocytes in the system in an hour. It can be seen from the table that the haemolytic effect is greatly increased by the action of Na thioglycolate and/or ascorbic acid. Obviously, the haemolysin is activated in reductive media. The lytic effect is completely suspended by 0,1 M of hydrogen peroxyde, which means that the lysin is sensitive to oxidizing agents. The activity of haemolysin suffers no reduction when associated with cholesterol. It dissolves the erythrocytes of man, sheep and mice in the same measure. While 0,1 ml of staphylo alpha antitoxic serum inhibits the haemolytic effect in a considerable degree, an equal amount of beta antitoxic serum has no effect on the haemolysin.

Table I

The effe ct of some substances on the haemolytic activity of supernatants of chloramphenicol-resistan S. typhi cultures

Agents added to the haemolytic system (HS)	Haemolysing titre of supernatant						
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
HS *alone*	+	+	—	—	—	—	—
HS + *reducing, or oxidizing agents*							
a) thioglycolate (0,05M)	+	+	+	+	+	+	—
b) ascorbic acid (0,05M)	+	+	+	+	+	+	—
c) hydrogen peroxide (0,1M)	—	—	—	—	—	—	—
Cholesterol inhibition							
HS + reducing agent + cholesterol ..	+	+	+	+	+	+	—
HS + cholesterol	+	+	—	—	—	—	—
Inhibition by staphylo antitoxic serum							
HS + α antitoxin 0,10 ml + red. agent	—	—	—	—	—	—	—
HS + β antitoxin 0,10 ml + red. agent	+	+	+	+	+	+	—

Note. There has been no difference in the haemolytic titre when using mouse erythrocytes or red blood cells of sheep, or those of man.

Optimum haemolytic action is obtained at p_H 6,8 to 7,2 and at a temperature between 37 and 42° C. It is weaker at room temperature and if the supernatant is Seitz-filtered or held at 100° C for 5 minutes, it is lost altogether. The haemolysin becomes inactivated if stored in a refrigerator for more than 10 to 14 days.

All in all, the haemolytic agent produced by chloramphenicol-resistant *S. typhi* has been found to be a substance closely related and similar to the members of the group of staphylolysins ; in our opinion it is the *exoferment* of bacteria.

The effect exterted by the haemolysin on chloramphenicol was then tested. Adding different concentrations of the reduced haemolysin to a solution containing 20 γ/ml of chloramphenicol, the quantity of chloramphenicol present in the system after the lapse of 1, 13, and 24 hours, respectively, was determined. It was found that the lysin does not break up or destroy the chloramphenicol molecules.

The changes occurring in the agglutinability and antigenic structure of the resistant *S. typhi* strains was also examined, as compared with the sensitive strains. Preparing serial dilutions with Vi, O and H antigens of known titres, the differences in the agglutinability of sensitive and resistant strains were observed.

It was found that the Vi agglutinability of the resistant bacteria was decreased, while their O agglutinability was substantially increased ; H antigen caused no significant difference between the agglutination titres of the sensitive and resistant strains. The results of these experiments are illustrated in Fig. 2.

Thereafter the rate of growth of the sensitive and resistant strains was tested, using a densitometer for this purpose. Bacterial density was measured every hour in a total volume of 10 ml of broth culture inoculated with 0,1 ml

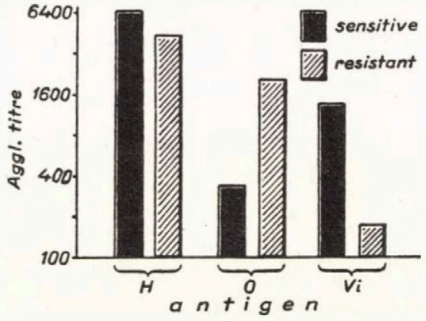

Fig. 2. Agglutinability of *S. typhi* strains sensitive and resistant, respectively, to chloramphenicol (mean values of 3 strains each)

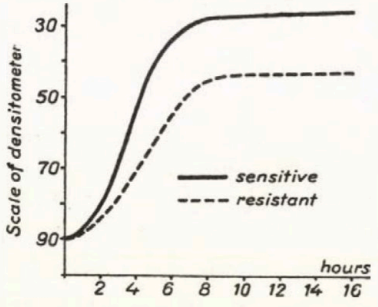

Fig. 3. Growth curves of *S. typhi* strains sensitive and resistant, respectively, to chloramphenicol (100 γ/ml)

of a 24-hour bacterial suspension. The results of the tests can be seen in Fig. 3 which shows the mean value of the growth curves of 4 sensitive and 4 resistant bacteria.

The curves reveal that in the resistant strains growth was considerably slower, the resting phase longer, the logarithmic growth section more flattened than in the sensitive strains, and that the final bacterial density was less than hat of the sensitive bacteria.

Discussion

There are sporadic references in the literature to a haemolysing activity exerted by *S. typhi* strains under certain conditions. We find, for instance, the following passage in GROMASHEVSKY's work [9] : «Evidence is accumulating that besides producing endotoxin, typhoid bacilli secrete also exotoxin which possessés a haemolysing capacity». Also, on rare occasions it was observed that among the strains freshly isolated from diseased organisms there occurred some of a haemolysing quality (RAUSS, [10]). Our observations of chloramphenicol-sensitive *S. typhi* strains kept under restricted conditions for prolonged periods of time, seem to indicate that these strains develop a haemolysing activity as a result of unfavourable life conditions, *e. g.* a slow

desiccation of the culture medium. There are also some «degraded» typhoid strains that possess this quality.

How is it, then, that observations show the haemolysing activity to have developed in all *S. typhi* strains that had been adapted to chloramphenicol? This is a significant phenomenon, since it is known that the enzymatic activity of bacteria such as dehydrogenase activity, sugar fermentation, production of exoferments etc., greatly diminished as they acquire resistance to antibiotics *in vitro*.

Chloramphenicol inhibits the normal metabolism of bacteria, which in the first phase of adaptation protect themselves against a blocking of their fermentative functions by altering their metabolism. Such alteration is then followed by a change of the bacterial cell surface. That there are surface processes at play is shown by the reduction of Vi agglutinability and the increased agglutinating effect of O sera on the resistant bacteria.

The change in the metabolism of the resistant bacterium reduces the efficiency of its energy-providing catabolic enzymes ; it may be said to pass into a phase of chronic starvation in which the metabolic activity, growth, and multiplication of the strain suffer a reduction as compared with those of the sensitive strains.

It is assumable that the inhibition of food intake and energy supply in the resistant bacterium is caused by a reduced production of adaptive ferments. The production of adaptive ferments implies the entry of the substrate into the cell (inducing agent) on the one hand, and on the other the presence of free amino acids for the development of enzyme proteins, as also a sufficient amount of energy.

It is not impossible that bacteria respond to the inhibition of their mechanism to produce adaptive enzymes with an intensification of the lytic ferment system. This might be characterised by the appearance of the haemolysing enzyme. It is just by the alteration of the cellular surface that this behaviour of the bacterium, which has changed its metabolism and intensified the production of lytic enzymes as a response to the inhibition of all other fermentative activities, becomes manifest and demonstrable. The haemolytic ferment appears as a result of the intensified production of the lytic enzymes preformed in the cells. This is confirmed by the fact that, to a limited extent, even the reduced supernatants of 48- to 72-hour cultures of sensitive strains are capable of dissolving erythrocytes. This is, however, never evident under normal conditions, and there is no need for it. It is only under the influence of inhibited and changed metabolism consequent upon the action of chloramphenicol, or other restricted life conditions *e. g.* during slow desiccation on a culture medium, that an increased production of the lytic ferment sets in.

Comparing these experimental results with our observations made on strains of *E. coli dyspepsiae* rendered resistant to chloramphenicol in vitro

(VÁCZI, GÁL and KUBINYI, [9]), it is interesting to note that these closely related bacteria responded to identical environmental influences with intesifying different types of fermentative activity.

While resistant strains of *E. coli dyspepsiae* produce an enzyme which destroys chloramphenicol (coli reductase), no such ferment is formed by resistant strains of *S. typhi*. The change undergone by their metabolism while they turn resistant, manifests itself in the production of an enzyme of a quite different type, and points to entirely different mechanisms in the metabolism of these bacteria belonging to two different species. Evidently, the response of a bacterium to the action of the identical antibiotic cannot forthwith be generalised. Identical action brings forth different changes in the fermentative activity of different species of bacteria, and it is only the result of these changes that remains the same throughout : it safeguards the subsistence of the bacteria in the changed environment.

Summary

1. The properties of *S. typhi* strains made resistant to chloramphenicol *in vitro* have been examined.

2. *S. typhi* strains adapt themselves rapidly to chloramphenicol. In from 4 to 6 weeks, they grow even on culture media which contain 100 γ/ml of chloramphenicol.

3. As regards their capacity to break up sugar and alcohol, there is no qualitative difference between sensitive and resistant strains of *S. typhi*.

4. On blood agar, resistant strains of *S. typhi* are intensively haemolytic.

5. The haemolytic titre of the supernatants prepared from resistant cultures is increased by reducing agents, blocked by oxidising agents, and uninfluenced by cholesterol. The erythrocytes of different species of animals, including mice, are dissolved by the same dilution of an active supernatant. The haemolytic agent is similar in its nature to the staphylolysin.

6. The haemolytic ferment is ineffective against chloramphenicol.

7. During the process of their turning resistant, the agglutinability of *S. typhi* strains with Vi serum is reduced, and with O serum increased.

8. The growth of resistant *S. typhi* strains becomes slow, the resting phase is prolonged, and the final bacterial density is inferior to that of sensitive strains.

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ГЕМОЛИТИЧЕСКИЙ ФЕРМЕНТ РЕЗИСТЕНТНОГО К ХЛОРАМФЕНИКОЛУ ШТАММА S. TYPHI

Л. ВАЦИ и И. МИХАЙФИ

Резюме

1. Авторы описывали свойства штаммов S. typhi, сделанных in vitro резистентными к хлорамфениколу.

2. Штаммы S. typhi быстро адаптируются к хлорамфениколу. Спустя 4–6 недель вырастают и в среде, содержащей 100 гамма/мл хлорамфеникола.

3. Не имеется качественного различия между чувствительными и резистентными штаммами S. typhi в отношении ферментации сахара и алкоголя.

4. Резистентные штаммы S. typhi обнаруживают сильное гемолитическое действие на кровяном агаре.

5. Восстановительные вещества усиливают, окислители тормозят эффект бактерийного лизина, холестрин не действует на него. Оно лизирует эритроциты различных видов животных, в том числе мышей, в одинаковом разведении. Литический фермент является экзоферментом, сходным по природе со стафилолизинами.

6. Литический фермент не действует на хлорамфеникол.

7. В процессе образования резистентности агглютинабильность штаммов S. typhi с Vi-сывороткой понижается, а с O-сывороткой повышается.

8. Размножение резистентных штаммов S. typhi замедляется, лаг-фаза затягивается, конечное число микробов меньше, чем у чувствительных.

9. Появление литической ферментативной активности является результатом приспособления резистентных бактерий, — с измененным характером обмена веществ — на базе обсужденного в дискуссии механизма.

ISOLATION OF COXSACKIE VIRUS STRAINS FROM INFECTIONS OCCURRED IN THE YEARS 1952—54 AND THEIR IDENTIFICATION

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Since DALLDORF and SICKLES had published their report on the discovery of Coxsackie (in the following: C) virus [1] an increasing number of data has been made available relative to the biological and physicochemical properties, behaviour under experimental conditions, and relationship to different human diseases of the single members of this group of virus. During the past seven years the various types of C virus have been shown to occur in most parts of the world and a number of reports have dealt with its occurrence in Europe. IVÁNOVICS and PINTÉR in 1952 were the first to report on the isolation of C virus in Hungary [2]. Later, PINTÉR and BALÁZS isolated and identified further 9 A-type strains of C virus in this country [3, 4]. The clinical aspects of the C infections confirmed by the above isolations were described by IVÁDY, PINTÉR and SZABÓ [5].

At the Department of Virology, State Institute of Public Health, the work on C viruses began in the autumn of 1952 and isolation experiments in test samples from hospitalised patients and single outbreaks have been carried out continuously ever since. Until December, 1954, 451 test samples from 426 individuals were examined by different methods, and from these 21 strains of virus pathogenic for suckling mice could be isolated. A certain proportion of the blood samples from human subjects was studied also by serological methods. In the present paper, the isolation experiments and the conclusions derived therefrom, as well as the work concerned with the identification of the 21 strains isolated will be described. In addition, an account will be given of clinical and epidemiological observations made in connection with this work.

Materials and methods

Virus isolation experiments. White mice, 1 or 2 days old were used, at least 8 animals from different litters for each isolation series. Each suckling mouse was given 0,03 to 0,04 ml of the adequately prepared test sample subcutaneously, in the interscapular area. After inoculation, each family was placed into a separate box and the sucklings were kept under observation for 10 days. Animals dead within 24 hours following inoculation were left unconsidered when evaluating the results. Samples from mice showing the symptoms of C infection and from mice which died later but have previously shown no sign of bacterial infection, were subjected to passage. The isolation experiment was repeated if after the second day disappearance due

to cannibalism occurred, as it was found that some mothers eat their sucklings at the onset of disease. Had the isolation experiment and the passage therefrom brought a positive result, the test samples were subjected to further, repeated isolation experiments. The strain was considered to be an original one only if it could be isolated repeatedly from test samples.

Particular attention has been devoted to the elimination of cross infection and intermixing of virus strains. While in use, the laboratory table was covered with gauze soaked in 0,5—1,0 per cent formaldehyde, chaking the gauze for each new test sample, At the end of each step in the work the table was exposed to ultraviolet irradiation for 30 minutes. The inoculated animals were kept in a chest containing boxes isolated from one another. While carrying out the control examinations, a carpet soaked in 0,5—1,0 per cent formaldehyde was placed on the floor before the chest in order to collect on it material that may have fallen out of the boxes. Before opening the door of a box and on completion of the control examination intensive hand disinfection was carried out. After the isolation experiments had been concluded, the boxes were cleansed with the above disinfectant.

Test materials. A 10 to 20 per cent suspension of faeces in physiological saline was centrifuged at 9000 r. p. m. for 30 minutes. To the supernatant were added penicillin and streptomycin, 1000 U and 2 mg per ml, respectively. If mice inoculated with the supernatant containing the above antibiotics showed signs of bacterial infection, aureomycin was also added and the experiment was repeated. Throat and ocular swabs received in diphtheria tubes, were washed from the cotton tampon with 1 to 2 ml of physiological saline and treated with antibiotics in the same way. Samples of cerebrospinal fluid obtained under sterile conditions were subjected to no pretreatment. Post-mortem specimens were homogenized with quartz dust, diluted with physiological saline to about 5 times the original volume and centrifuged at 9000 r. p. m. for 30 minutes. The supernatant was treated with antibiotics as described for faeces.

Preparation of immune sera. A 20 per cent suspension was prepared of the torso of newborn mice infected with virus. This was used to inoculate 50 five weeks old mice in each strain, by the intraperitoneal route, on four occasions. At intervals of four days three doses of 0,25 ml were injected, the fourth inoculation was carried out 14 days after the third one, this time with 0,5 ml. Two weeks after the fourth inoculation the animals were killed by bleeding. The immune sera were divided into 0,5 ml doses and stored after lyophilization. Before use the samples were diluted to 5 times the initial volume and inactivated by maintaining at 60° C for 20 minutes.

Preparation of complement fixing antigens. A number of mice not more than 1-day old were infected with a 20 per cent suspension of the torso of mice infected with virus. If at least 50 per cent of the test animals developed symptoms of disease within 36 to 48 hours after inoculation, all animals were killed by chloroform. In single cases bleeding under chloroform was also employed. After removing the viscera, head, paws and skin, the torsos were homogenized with quartz powder in a mortar and diluted to 4 times the initial volume with physiological saline. After storing overnight in a refrigerator, the samples were thawed and centrifuged at 4000 r. p. m. for 15 minutes. The supernatants were purified mostly by the protamine sulphate precipitation method [6, 7]. Where this method failed to yield antigen of required strength, the same sample was subjected to freezing and thawing. The protamine sulphate method was carried out as follows. To each ml of the crude torso suspension were added 4 mg of protamine sulphate. The mixture was thoroughly shaken in a well-closed glass flask until a rough precipitate had formed, which was then removed by centrifugation at 4000 r. p. m. for 15 minutes. The freezing-thawing method was carried out as follows. The suspension was frozen by submersion into a mixture of dry ice and methanol, thawed 6 times in succession by submersion into tap water and finally centrifuged at 9000 r. p. m. for 20 minutes. The protamine sulphate method yielded more stable antigens. The methods used for the preparation of normal antigens from torsos of apparently unaffected mice were the same.

Complement fixation test. Essentially, the technique was that described by TAKÁTSY [8a, b]. Dilutions of serum were prepared in grooved plexiglass plates, using the TAKÁTSY's 0,025 ml spiral loop. The mixture of antigen and complement was prepared in advance and was adjusted to contain 2 units of antigen and 2 units of complement. Of this, 0,025 ml were added to each dilution of serum in the grooves. After allowing it to stand in an incubator of adequate humidity at 37° C, 0,025 ml of the haemolytic system containing 8 units of haemolysin and 3 per cent sheep erythrocytes were added to each dilution. Readings were taken after an additional 20 minutes of incubation. Dilutions of sera are given in terms of final concentration of the complement fixing system.

Neutralization test. A dilution of virus containing 200 LD50 in each 0,03 ml was mixed to equal volumes of different serum dilutions and after standing for 1 hour at 4° C the inocufations were carried out, injecting 0,03 ml into each animal. The LD50 was determined by the lollowing method. The viral suspension was diluted with physiological saline containing normal

rabbit serum in tenfold steps and with each dilution a litter of mice containing 8 sucklings was inoculated, injecting 0,03 ml into each mouse. Evaluation was made on basis of REED and MUENCH's formula [9].

Storage of virus strains. 20 per cent suspensions of muscle of animals infected with standard and isolated strains were lyophilized and stored at +4° C. The samples lost practically none of their activity over periods extending to 1½ years.

Experimental

Grouping of test series. In the period from October, 1952, to December, 1954, a total of 451 samples from 426 subjets was examined for presence of virus. Samples of faeces from 391 subjets, faeces and throat swabs from 20, faeces and post-mortem brain specimens from 3, blood, brain and colon content from 1 autopsied case, CSF from 4 subjects and post-mortem brain specimens from 7 subjects were studied. Most of the test samples had been sent in by physicians in constant contact with us. Being familiar with the pertaining literature, they could reliably select the cases suspect of C infection. Sometimes the Department of Epidemology of this Institute had called attention to outbreaks suspected of being caused by C infection; in such instances we ourselves visited the affected area and collected the test samples. In addition, cases and outbreaks of unknown aetiology, which, however, on the basis of the literature could not be suspected of being due to C infection, were also examined. Such diseases or outbreaks included encephalitis, haemorrhagic nephrosonephritis, stomatitis, etc. and are shown in Table I in the column «Others». In the group «Suspected C infection» are included the patients whose samples were sent in by the physicians as suspect, without any further information about the symptoms. Moreover, in 1954, 74 samples of faeces from patients with poliomyelitis were collected and examined.

Distribution of positive cases. The test samples or specimens originated from various areas of the country. Relatively many were received from Miskolc and its surroundings, as well as from Budapest. The diagnoses in Table I are those suggested by the physicians sending in the specimens.

A total of 21 strains of virus pathogenic for suckling mice were isolated, all from faecal samples. Excretion of virus could be demonstrated in 2 of 31 patients during the last quarter of 1952, in 14 of 197 in 1953, while in only 5 out of a total of 198 patients in 1954.

In none of the cases did we succeed in recovering C virus from patients with aseptic meningitis, from any healthy person living in contact with patients suffering from poliomyelitis, or from patients included in groups «Others» and «Suspected C infection».

It is remarkable that all the 13 specimens originating from patients with a diagnosis of Bornholm disease were negative. The strain designated with an asterisk was isolated from a case of laboratory infection.

Only 1 of the 11 cases of herpangina proved to be positive.

Table I

Number of patients examined, incidence of recovery of virus in different clinical diseases

Diagnosis	Year of testing			Total
	1952	1953	1954	
Bornholm disease	0/4	1/7*	0/3	1/14
Aseptic meningitis	0/2	0/6	0/45	0/53
«Three-day fever» Summer grippe Gastric grippe	—	9/93	3/25	12/118
Herpangina	0/3	0/4	1/4	1/11
Enteritis	2/6	1/12	0/22	3/40
Suspected C infection	0/6	0/33	0/9	0/48
Summer grippe in environment	—	3/6	—	3/6
Poliomyelitis (P & NP)	—	0/4	1/74	1/78
Poliomyelitis in environment	0/4	—	0/9	0/13
Others	0/6	0/32	0/7	0/45
Total	2/31	14/197	5/198	21/426

In the numerator the number of test subjects positive for virus, in the denominator the total number of test subjects are shown.

* Laboratory infection.

Most strains were recovered from faecal samples of patients diagnosed to have gastric grippe, summer grippe, three-day fever, enteritis or relapsing dysentery and from apparently healthy persons living in contact with them.

Only 1 of a total of 74 faecal samples from patients with poliomyelitis proved to contain virus.

The age incidence for 425 patients (the single case of laboratory infection has been omitted) is shown in Table II.

Table II

Age incidence among test subjects

	Age in years				
	< 2	2	3—5	6—15	> 15
Number of patients examined	110	61	55	68	131
Number of virus positive test subjects	5	11	2	1	1
Positivity, per cent	4,5	18,0	3,6	1,5	0,8

Table III

Data for the 21 virus positive test subjects

Designation of strain isolated	Age of patient	Date of receiving specimen	Diagnosis	Community
6. KT.	2½ yrs.	Sept. 11, 1952	Dysentery relapse	Jánoshalma
7. SzL.	4 months	« « «	« «	«
33. UM.	2 yrs.	Jan. 10, 1953	»Three-day fever«	Budapest
74. MK.	2 yrs.	June 26	Summer grippe in environment	«
75. GI.	2½ yrs.	June 26	Summer grippe in environment	«
81. KT.	2 yrs.	June 26	Summer grippe in environment	«
98. MJ.	54 yrs.	July 10	Enteritis	Jánoshalma
116. LT.	10 months	July 15	Gastric grippe	Miskolc
119. NÉ.	11 yrs.	July 15	« «	«
120. ZK.	2½ yrs.	July 15	« «	«
124. OÉ.	15 months	July 15	« «	Miskolc area
130. OJ.	5 yrs.	July 20	« «	« «
139. FL.	2 yrs.	July 21	« «	Miskolc
153. HL.	2 yrs.	August 2	« «	Miskolc area
178. TJ.	6 months	Aug. 19	« «	« «
270. DM.	30 yrs.	Dec. 7.	Pleurodynia*	Budapest
308. NF.	9 months	March 13, 1954	Poliomyelitis (P)	Ózd
331. KZs.	2 yrs.	June 27	Herpangina	Budapest
479. OJ.	4 yrs.	Sept. 25	Gastric grippe	Miskolc area
495 KS.	2 yrs.	Oct. 1	« «	« «
497. IB.	2 yrs.	Oct. 1.	« «	« «

* Laboratory infection.

226, i. e. 53 per cent of the total number of patients tested, were from 2 weeks to 5 years of age. Of these, 18, i. e. 7,9 per cent were positive. A more detailed analysis of the age group under 5 years reveals that the largest number of strains was isolated from faecal specimens of 2-year old patients. It should, however, be emphasized, that the 2-year old virus carriers lived in different areas of the country and were affected at different points of time (*v.* Table III).

In Table III are shown the data of those 21 subjects from whom the strains of virus were isolated.

Clinical and epidemiological data. The first strains were isolated from faecal specimens of two children living in Jánoshalma. In this small village a small-scale outbreak of an influenza-like epidemic was observed in July, 1952, followed in August by an explosive epidemic of entero-colitis affecting 1/3 of the population, that lasted 6 weeks. Early in September an outbreak of poliomyelitis occurred. Clinically, most cases of enterocolitis were diagnosed as dysentery and from a considerable proportion of patients *Sh. flexneri* could be actually recovered. There were, however, mostly among infants and children disease patterns not resembling dysentery either clinically or bacteriologically. Some of these patients apparently recovered after fever and diarrhea had after a few days subsided, but in many a case the short symptom-free period was followed by another outbreak of fever and recurrence of enteral complaints. Many of these children were admitted to the hospital, exhibiting symptoms including high fever, exsiccation, somnolence, intractable vomiting. Faecal specimens were obtained from 4, freshly affected children in the period when in Jánoshalma the poliomyelitis outbreak was in its initial stage. All four specimens proved bacteriologically negative, while from two of them C virus could be recovered. Unfortunately, technical difficulties prevented us from visiting the affected area earlier than 3 months after the onset of the epidemic and thus no detailed epidemiological study has been carried out. Retrospectively, it appears to be likely that the summer grippe epidemic that had broken out in July took a prolonged course and gastric forms of that disease still occurred at the time the dysentery epidemic was in progress.

Strain 33. UM. was recovered from a child under treatment in a tuberculosis sanatorium in Budapest. In that sanatorium a domestic outbreak affecting 2 wards occurred early in January, 1953. A total of 8 samples of faeces from the affected children was examined. The disease pattern was characterized by high fever for 2 to 3 days, sore throat, and weakness. In most cases the actual disease did not last longer than a few days, but in some instances superinfection caused a flare-up in the tuberculotic process, with two fatal cases among the patients. Unfortunately, by the time the latter became known to us, it was too late to carry out a post-mortem examination for the presence of C virus.

Strains 74. MK., 75. GI. and 81. KT. originated from the nursery of a Budapest social home. At the time the specimens were obtained, all three children were apparently healthy; however, one week before taking the samples an outbreak of summer grippe occurred in that home, followed by sporadic cases throughout the summer. The disease pattern was similar to that described above, but in some cases atypical cutaneous rashes and enteral symptoms also developed. Most patients recovered rapidly, but some had to be hospitalized because of meningeal symptoms. Faeces of two diseased and 6 apparently healthy subjects were examined. The virus could be recovered from the faeces of 3 of six normal, but from none of the affected subjects.

Strain 98. MJ. is the only one recovered from an adult patient, an inhabitant of Jánoshalma. He had enteritis (10 to 15 mucous stools daily) and high fever over a period of 3 days. Bacteriologically, the faeces from this patient were negative.

The next 8 strains in Table III were isolated from children living in Miskolc and in the Miskolc area. All samples were sent in by the Department of Paediatrics, Miskolc Hospital. From early in July, 1953, on, an increasing number of patients was admitted to that hospital with a disease diagnosed by Dr. Kostyál, chief Physician, as «gastric grippe». The disease pattern was characterized by a sudden onset with high fever (39° to 41° C), intense headache, conjunctivitis; throat complaints were uncommon. Meningeal symptoms were recorded in some cases. Usually on the third day of illness, vomiting set in, that could be relieved only by gastric lavage, insulin or dextrose administration. In some cases a rash also occurred. In single cases the temperature curve was biphasic, with an afebrile interval of 2 to 3 days between the two febrile periods. According to the hospital staff and physicians in general practice, a great number of similar cases had occurred in the Miskolc area in the same period of every year beginning rom 1951. Strains 478. OJ., 495. KS., and 497. IB. were recovered from similar cases in September fand October, 1954.

Strain 270. DM. caused typical pleurodynia in one of our colleagues. Five days before he had been working with the standard strain B3 and had presumably suffered a respiratory infection when preparing the viral suspension (homogenization of mouse torsos), as he had been wearing no mask or protective glasses. The onset of the disease on December 7, 1953, was marked by depression, headache and nausea. On December 8 he developed in the left epigastrium a very intense sharp pain which increased on breathing, motion and compression of the chest. The pain was located to the left half of the diaphragm. One day later the right diaphragm was also painful, and the patient complained of vague pains in the thighs. The nasopharyngeal mucosa was inflamed. A chest X-ray on that day showed slightly impaired diaphragmatic motion, but otherwise nothing abnormal. Pain subsided in about 1 week. The temperature curve was biphasic, with high fever on the first two days, followed by an afebrile period of 3 days' duration, and another episode of high fever on the fifth day of illness. Faecal samples taken 2 and 9 days following the onset were examined for presence of virus. Virus could be recovered from both specimens, in spite of the fact that at the time when the second specimen had been taken the patient had no symptoms any more.

The only case of poliomyelitis positive for C virus was that of an infant in a nursery. In March, 1954, two cases of paralysis occurred simultaneously in that nursery. From the faeces of one patient strain 308. NF. was isolated. At that time, poliomyelitis morbidity was at the average level in Hungary. Virus pathogenic for suckling mice could not be isolated from the faeces samples of patients developing poliomyelitis during the epidemic period from July until October.

Strain 331. KZs. was recovered from the faeces of a patient under treatment at the Pediatric Dept. of a Budapest hospital. The patient had been admitted two days after the onset of a disease diagnosed as febrile ecclampsia and herpangina. After a febrile period of 5 days' duration, the patient made a complete recovery.

In Table IV are shown the annual and monthly distributions of test samples.

Table IV

Date of onset of disease in patients tested

Month \ Year	1952	1953	1954	Total
January	—	1/17	0/14	1/31
February	—	—	0/2	0/2
March	—	—	1/22	1/22
April	—	—	0/1	0/1
May	—	—	0/4	0/4
June	—	3/8	1/8	4/16
July	—	7/87	0/40	7/127
August	—	2/29	0/32	2/61
September	2/9	0/29	1/45	3/83
October	—	0/13	2/26	2/39
November	0/7	0/3	0/4	0/14
December	0/15	0/10	—	0/25

Numerator: Number of virus positive patients.
Denominator: Total number of subjects tested.

As seen, the incidence of cases suggesting C infection was the highest in the period from June until September. The number of isolations was also the

highest during that period. The seasonal incidence is in agreement with data in the literature. The examinations were begun in 1952, at the end of the epidemic period. The greatest number of test samples was received in June, July and August, 1953, and 11 of the 12 strains were recovered from epidemics. The situation was different in 1954. Our connections with physicians in different parts of the country having already been well established, we expected to receive a great number of test samples. Still, the number of samples sent in during the summer and early autumn was considerably lower than in 1953. The difference between the two years becomes even more conspicuous if only such cases are taken into consideration in which on the basis of clinical diagnosis C infection was suggested and cases of paralytic poliomyelitis and those listed in the group «Others» in Table I are omitted (Table V).

Table V

Incidence of illnesses suspicious of C virus infection during summer and early autumn

Month \ Year	1953	1954
June	3/8	1/5
July	7/71	0/18
August	2/27	0/21
September	0/20	1/32
October	0/13	2/11
Total	12/139	4/87

Numerator: Number of virus positive subjects.
Denominator: Total number of test subjects.

It is remarkable that the relative number of strains isolated in 1954 was also smaller. In the summer and early autumn months of 1953 the incidence of positive cases was 8,9 per cent (12 cases) while in 1954 only 4,6 per cent (4 cases). The decrease in the number of test samples received and in the frequency of successful isolations, as compared to the data for 1953, indicates that early in the summer of 1954 the usual increase in the incidence of C infections for some unknown reason did not take place.

Identification experiments

The 14 standard strains (Types 1 to 10 of the A-group, and types 1 to 4 of the B-group) obtained in 1953 from *G. Dalldorf* were used for identifying our strains. In the first step mice were immunized with each of the 14 standard strains. Homogeneity of the standard strains was proved by testing against the

immune sera prepared. The purity of the strains was confirmed by both cross neutralization and complement fixation tests. In Table VI the results of complement fixation tests made with the standard strains are also included.

Table VI

Identification of new strains by complement fixation tests

Antigens	Mouse Immune Sera														
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	B1	B2	B3	B4	130
A1	*160	—	—	—	—	—	—	—	—	—	—	—	—	—	X
A2	—	160	—	—	—	—	—	—	—	—	—	—	—	—	X
A3	—	—	160	—	—	—	—	—	—	—	—	—	—	—	X
A4	—	—	—	160	—	—	—	—	—	—	—	—	—	—	X
A5	—	—	—	—	80	—	—	—	—	—	—	—	—	—	X
A6	—	—	—	—	—	80	—	—	—	—	—	—	—	—	X
A7	—	—	—	—	—	—	40	—	—	—	—	—	—	—	X
A8	—	—	—	—	—	—	—	160	—	—	—	—	—	—	X
A9	—	—	—	—	—	—	—	—	160	—	—	—	—	—	X
A10	—	—	—	—	—	—	—	—	—	80	—	—	—	—	X
B1	—	—	—	—	—	—	—	—	—	—	80	—	—	—	X
B2	—	—	—	—	—	—	—	—	—	—	—	80	—	—	X
B3	—	—	—	—	—	—	—	—	—	—	—	—	160	—	X
B4	—	—	—	—	—	—	—	—	—	—	—	—	—	80	X
6. KT.	—	—	—	—	—	40	—	—	—	—	—	—	—	—	—
7. SzL.	—	—	—	—	—	40	—	—	—	—	—	—	—	—	—
33. UM.	—	—	—	—	—	—	—	160	—	—	—	—	—	—	—
75. GI.	—	—	—	80	—	—	—	—	—	—	—	—	—	—	—
81. KT.	—	80	—	—	—	—	—	—	—	—	—	—	—	—	—
116. LT.	80	—	—	—	—	—	—	—	—	—	—	—	—	—	—
119. NÉ.	—	—	—	80	—	—	—	—	—	—	—	—	—	—	—
120. ZK.....	—	—	—	—	—	—	—	—	160	—	—	—	—	—	—
124. OÉ.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	320
130. OJ.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	320
178. TJ.	—	—	—	—	—	—	80	—	—	—	—	—	—	—	—
308. NF.....	—	—	—	160	—	—	—	—	—	—	—	—	—	—	—
270. DM.	—	—	—	—	—	—	—	—	—	—	—	—	80	—	—
270/a. DM.	—	—	—	—	—	—	—	—	—	—	—	—	80	—	—

* Reciprocals of complement fixing titres.

— = negative in dilution 1 : 4

X = not tested.

Identification of our own strains was made by the complement fixation method, except strains 6. KT., 7. SzL. and 130. OJ., which were tested by the neutralisation method, too. In the complement fixation tests the antigens prepared from our strains were matched with each one of the standard sera. In general, the strains had to be subjected to 4 to 5 passages in suckling mice to yield antigens of desired potency. So far, 13 strains could be identified, of which 1 proved to be identical with the Al, 1 with the A2, 3 with the A4, 2 with the A6, 1 with the A7, 1 with the A8 and 1 with the A9 types of Dalldorf, while 2 strains could not be identified by testing against any of the 14 standard sera. As anticipated, from both faecal specimens from the subject with laboratory infection strain B3 was recovered. Of the 2 strains not identifiable by complement fixation, one (strain 130. OJ.) was examined by neutralization tests, too, but even so it could not be identified. It is thus obvious that strain 130. OJ. either belongs to a type of C virus differing from the 14 standards in antigenic structure, or does not belong to the C virus. Paralytic disease similar to that due to C virus may be caused in suckling mice by herpes virus or by the virus of mouse encephalomyelitis. As it proved to be resistant to ether and non-pathogenic for adult mice either intracerebrally or subcutaneously, strain 130. OJ. could not be identified with either of the latter viruses. As determined in complement fixation tests, the immune serum produced with this strain reacted positively not only with the homologous strain, but also with strain 124. OÉ. Thus, these two strains represent identical antigenic types, different from those of the Dalldorf strains. Apart from the 14 standard strains in our possession two more strains representing distinct antigenic types (strains Texas 12 and Texas 14) are known to exist [10]. Our non-identified strains might be identical with either of the latter. It is also possible, however, that we have succeeded in isolating strains of hitherto unknown type in Hungary.

Discussion

The primary aim of the present work has been to obtain detailed information on the extent of C infections in Hungary. Unfortunately, the complement fixation test is not the method of choice for this purpose [11, 12, 13] and, considering the existence of 16 antigenic types, neutralization test is a complicated procedure. It is also possible that antigenic types other than those already known exist and, obviously, they cannot be identified by serologic methods. In view of these considerations we have made chiefly isolation experiments.

According to data in the literature [14, 15], laboratory cross infections are common with strains of C virus. For this reason it had first to be decided whether the new strains isolated had actually originated from the patients examined. In the case of the two strains isolated in 1952, and of the two differing from the standards, the possibility of admixture could be excluded, since at the

time the former were being isolated no other strain was present in the laboratory, and the two latter strains were different in antigenic structure from the other strains available. When judging the originality of the rest of the strains, the circumstances of isolation should be considered. The precautionary measures taken have been described in the section dealing with the methods employed. As a further safeguard, we did not work with laboratory strains at the time the isolation experiments were performed. Each strain could be recovered repeatedly and the incidence of death in the second, or even third, series of experiments was comparable to that in the first series. A further proof of the originality of our strains is seen in the fact that with our strains usually 4 to 5 passages were required to obtain complement fixing antigens, as compared to the 1 or 2 passsages needed by the standard strains which had been properly adapted to suckling mice. It is further noteworthy that the results of serological tests made with human sera also pointed to the originality of our strains. An account of these tests will be published in another paper.

The question arose, what accounts for the fact that the presence of virus could be demonstrated in not more than 5 per cent of the patients examined? This may be due to a variety of causes. A great proportion of the test specimens originated from pediatric departments, where patients are usually admitted later than the first few days of the disease, when the optimum time for virus isolation is already over. It has also been mentioned that the cases examined included affections of unknown aetiology, which, on grounds of literary evidence, could not be suspected to be associated with infection by C virus.

According to the data in Table V, in the summer of 1954 the incidence of suspected C infections was lower than in the preceding year, and in 1954 we could isolate relatively fewer virus strains than in 1953. It has been suggested that this may have been in some way related to the 1954 epidemic of poliomyelitis. In June of that year a grave and prolonged poliomyelitis epidemic broke out in Hungary. The morbidity rate in each of the last six months of 1954 was much over the corresponding medians for the same months in 1946—52.

The relationship between C and poliomyelitis viruses has been frequently dealt with in the literature. The C virus had first been isolated [1] from children suffering from poliomyelitis, from whose faeces poliomyelitis virus could later be recovered [15]. Since then, numerous reports have been published on the simultaneous presence of the two viruses in the faeces of poliomyelitic and from apparently normal subjects. The mode of spread and seasonal incidence of the two viruses are identical. From this it follows that their simultaneous presence in the same individual may be coincidental, but one cannot exclude the possibility that one virus creates conditions favourable for the other and that the two viruses together might produce more serious disease. Finally, the contrary of this last possibility may also be true. In our opinion, any of the three possibilities may occur, depending on the actual general condition of the macroorganism,

on the type and quantity of viruses causing the infection, on the chronological relations of the invasion by viruses, and possibly on other factors. The literary data on the relationship between the two viruses are also conflicting. This applies to animal experiments and epidemiological observations alike. No interference or synergism between the two viruses could be demonstrated by MELNICK in mouse and monkey experiments [16], by STANLEY in monkey experiments [17], and by LEVADITI and VAISMAN in mouse experiments [18]. In mouse experiments, DALLDORF [19], STANLEY [17], SULKIN, and MANIRE [20], as well as SULKIN, WALLIS and MURPHY [21] showed that in simultaneous infection the interaction between the two viruses was partly inhibitory, partly enhancing, and in single cases, reciprocal in nature.

Although there is some epidemiological evidence suggesting interaction [22, 23, 24], other data appear to discredit this possibility.

In Hungary, PINTÉR and BALÁZS [3] have found a parallelism between C virus infections and poliomyelitis morbidity in Szeged. Their isolation tests had been namely unsuccessful before 1952, when the incidence of poliomyelitis had been also low, while in 1952, when they isolated strains of C virus, poliomyelitis morbidity was far above the average in Szeged. Similar conclusions may be drawn from our observations made in Jánoshalma in the year 1952. It appears that in that village a mixed epidemic involving C infection and dysentery had prepared the ground for the subsequent local outbreak of poliomyelitis.

On the other hand, the observations made in 1954 suggest an antagonism between the two viruses. In that year, the poliomyelitis epidemic had broken out in June and from that time on no C virus could be recovered from the faeces of patients with poliomyelitis, the incidence of C infections decreased, and the number of strains isolated was lower than in the previous year. These conflicting data emphasize the importance of systematic investigations into the relationship between the two kinds of virus.

PINTÉR and BALÁZS [4] have shown that types A4, A5 and A6 occurred in Hungary. We could demonstrate that, in addition to these types, also types A1, A2, A7, A8, A9 as well as an antigenic type different from the 14 DALLDORF standard strains cause disease in this country. Although the number of strains isolated in Europe is very high, only a few of them have been identified. In the reports available to us, the occurrence of the following types has been published : In Sweden, Strains A4, A5, A8, B3 [25, 26, 27] ; in Finland, A10 [28] ; in Germany, A2 [29] ; in Switzerland, A3 [30] ; in the Netherlands, B1 [31] A1, A2, A3 [32] ; in France, A1, A2 [10] ; in Czechoslovakia, A2 [33]. We could show that strains of the A7 and A9 type also occur in Europe.

We have been unable to demonstrate any relationship between the single antigenic types and the clinical pattern of the infection, since, for example, from patients with summer grippe strains A1, A2, A4, A7, A9, as well as the strain exhibiting a new antigenic type could be recovered.

Summary

In the period 1952—54, strains of virus pathogenic for suckling mice could be recovered from 21 out of a total of 451 test specimens examined. The test specimens originated from different parts of Hungary.

Isolation was successful in the highest proportion of cases diagnosed as summer grippe, but strains could be isolated also from patients with herpangina and poliomyelitis. In a case of accidental laboratory infection the symptoms were typical of Bornholm disease. From the faeces of the latter patient B3 virus could be isolated on two occasions.

The majority of strains were recovered from patients about 2 years of age. The affections described, and the minor outbreaks, occurred mostly in summer or in early autumn, but a few sporadic cases of C infection could be demonstrated in other parts of the year as well.

So far, 13 of the total of 21 strains could be identified. The strains belong to 8 antigenic types of which one differs from the 14 types available for us. The occurrence in Europe of 3, and in Hungary of 6, of these antigenic types was demonstrated for the first time.

The significance of a decrease in the number of C virus isolations during a nationwide poliomyelitis epidemic has been discussed.

Acknowledgements

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ИЗОЛЯЦИЯ ШТАММОВ ВИРУСА КОКСАКИ ИЗ ЗАБОЛЕВАНИЙ, СЛУЧИВШИХСЯ ЗА ПЕРИОД 1952—1954 Г. И ИХ ИДЕНТИФИКАЦИЯ

И. ДЭМЭК

Р е з ю м е

Автор за период 1952—1954 года из 451 исследовательского материала в 21 случае выделил штамм патогенного для мышат-сосунков вируса. Исследовательский материал происходил из различных территорий Венгрии.

Эксперименты оказывались успешными наиболее часто в тех случаях, где клинический диагноз был летний грипп, но удалось изолировать штаммы также и от больных герпангиной и полиомиэлитом. Случайная инфекция у лабораторного работника вызвала типичную боргольмскую болезнь. Из стула больного в двух случаях удалось выделить штамм вируса типа B3.

Большинство штаммов происходит из возрастной группы около двух лет. Описанные заболевания и мелкие эпидемии появлялись в летних и раннеосенних месяцах, но и в другие периоды года несколько спорадических случаев также оказались заболеваниями Коксаки. Во время повсеместной эпидемии полиомиэлита частость выделения вируса Коксаки снизилась.

До настоящего времени автор идентифицировал 13 штаммов с помошью 14 стандартных штаммов Дальдорфа (A1— 10, B1— 4). Штаммы относятся к 8 антигенным типам (A1, A2, A4, A6, A7, A8, A9), из которых один отличается от типов Дальдорфа.

NOTES ON THE MICROFLORA OF SALAMI AND ON ITS CONTROLLED DEVELOPMENT

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Salami sausage is a meat product prepared from raw meats by long maturation, *i. e.* slow drying without the application of heat or of other more powerful germicidal agents. Thus, many microorganisms may be found in the product and on its surface as well. This is especially true in the case of the so-called Hungarian salami, the surface of which is covered by a characteristic greyish- or yellowish-white mould layer developed during periods of drying and storing, generally much longer than usual in other countries. Nevertheless, no publication relating to the microflora of salami could be encountered in the literature. Even the chemistry and technology of salami manufacture are dealt with in only a few articles. It has, therefore, been decided, to study some aspects of the microflora of salami.

Details of one part of our experiments have been published earlier [3]. The present paper discusses mainly the microflora of the surfaces. Results of work on the internal flora will now be reviewed very briefly.

It has been found that the *microflora of the inner portions* of salami sausage chiefly consists of cocci and rod-like bacteria many of which are proteolytic in nature. Their numbers are subject to substantial changes in the course of processing operations. The bacterial count of the raw material increases during removal of the bones and chopping. This rise continues in the initial stages of drying. Then, from the 50th or 60th day onwards, the number of viable cells slowly decreases. The decline in bacterial count is connected with the fact that, as drying proceeds, the salts of the paste — expecially the NaCl added — are present in solutions of ever increasing concentration [1]. Thus, the «Hydratur» [5] of the product steadily decreases, and conditions of microbial growth gradually deteriorate, leading ultimately to the death of the cells. The antimicrobial action of spices added to the paste may hasten this process.

As regards the question whether the inner microflora of salami plays an active part in the development of flavour, experiments were carried out to suppress microbial activity in the paste by adding chemicals to it before filling into casings. Organoleptic properties of sausages prepared in this way and by the normal procedure (without chemicals) were compared. Unfortunately,

since no efficient preservative, harmless to human health, could hitherto be found, only partial destruction of bacteria could be achieved. Even so, we have succeeded in making it probable that the effect of the internal microflora on flavour was not a decisive one.

Examination of *micro-organisms on the surface of* Hungarian salami led to the conclusion that, in the course of development of the «cover», first yeast-like organisms *(Torula*, etc.) and bacteria appear. After a few weeks of drying, the latter are overgrown by the moulds characteristic of Hungarian salami. According to air analyses, all of these microorganisms reach the fresh sausage from the air of the drying room.

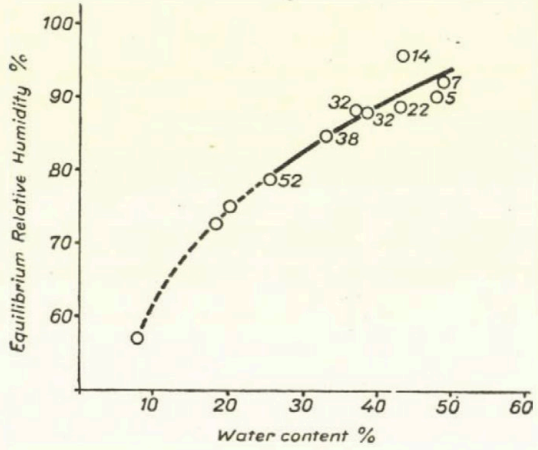

Fig. 1. Figures at the points indicate the duration of drying (in days) Equilibrium relative humidity values below 75 per cent were observed only in samples of sliced salami kept for longer periods in the refrigerator

Development of the mould cover depends on several conditions. Besides temperature, relative humidity of the air is of prime importance. In too dry a room *(e. g.* under 70 per cent relative humidity) the cover, if at all, does not develop satisfactorily. If, on the other hand, the space is too damp *(e. g.* above 85 per cent relative humidity), slime-forming bacteria, yeasts and moulds producing unpleasant odour or flavour may suppress the desirable micro-organisms. — This qualitative picture about the development of microflora on the casings may well be explained by a progressive decrease during drying in that chief factor governing the sort of appearing micro-organisms *i. e.* in the equilibrium relative humidity of the sausage substance. This decrease had been studied from another angle in earlier experiments [2]. A batch of fresh salami had been examined during normal drying. After various periods of ripening, samples had been taken from the batch and their water content and equi-

librium relative humidity determined by a modification of VINCENT and BRISTOL's method [4] based on vapour pressure measurement. As seen from Fig., 1., equilibrium relative humidity of the fresh sausage starts with a value of around 90 per cent and decreases to about 80 per cent in the «ready to cut» state. According to the pertaining literature and to our own experience, bacteria usually do not grow under an equilibrium relative humidity of 95 per cent, yeasts under 90—91 per cent and moulds below 75 per cent. In this way it becomes understandable why, in the first stages of drying, quickly

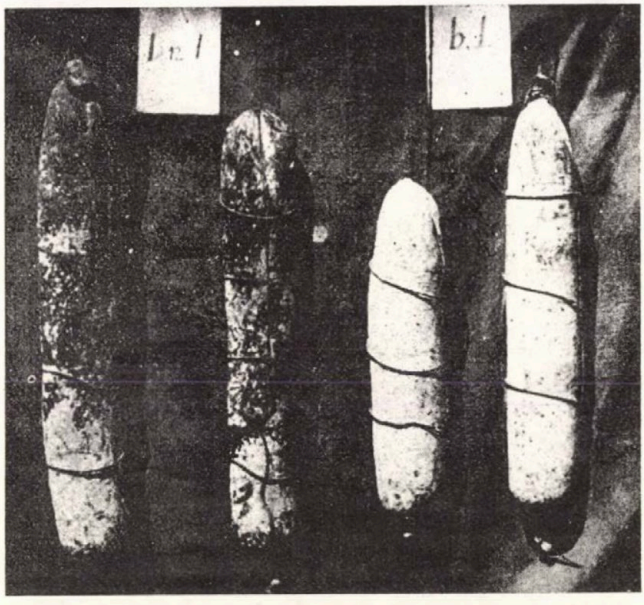

Fig. 2. b. 1.: Salami inoculated with *Aspergillus candidus* (after one month of drying)
bn. 1.: Control, without artificial infection (after one month of drying)

growing yeasts appear and why, later on, these are superseded by moulds more resistant to dryness.

In our report cited above [3], which contains the experimental details, we had also tried to establish how the development of flavour is influenced by the external microflora. Organoleptic properties of Hungarian salami covered by the usual mould layer and of mould-free samples of the same age had been studied by triangular taste tests. Statistical analysis of the data revealed that the flavour of the sausage is not influenced by the mould layer *directly*, in that it would produce some characteristic flavour component. The outside layer may, however, exert an *indirect* influence, as cover-formation by the «mould of choice» prevents the growth of harmful moulds and bacteria which would lead to mustiness or other undesirable changes in flavour. Similarly, the mould

cover may exert a favourable indirect influence on maturation by slowing down the rate of drying and thus making ripening of the sausage more uniform.

On the grounds of the above experience, it is thought probable that development of the characteristic flavour and aroma is caused — besides the spices — by enzymes functioning in the meat tissue. Micro-organisms play an indirect

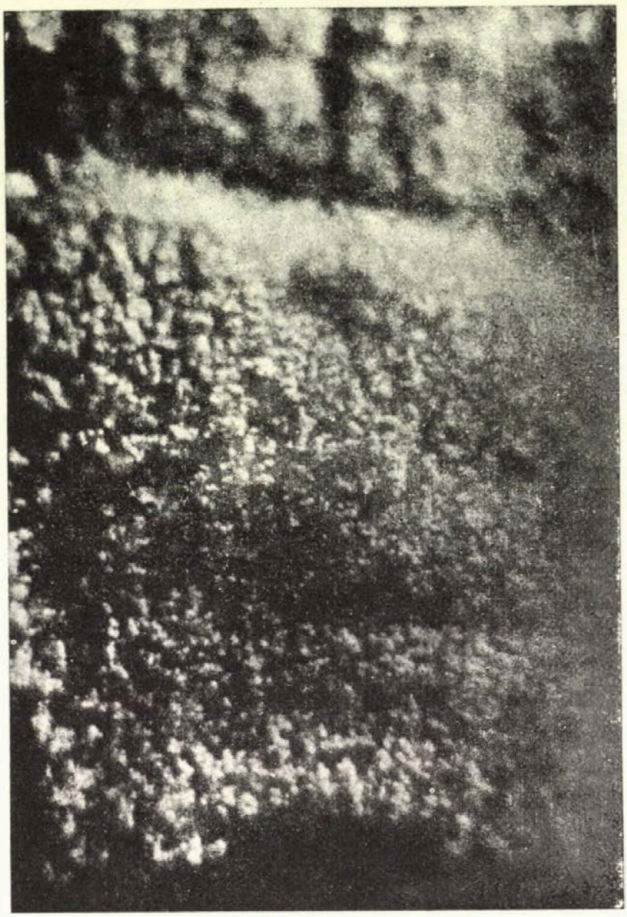

Fig. 3. Cover formed by *Aspergillus candidus* on the surface of salami (magnified about 50 fold)

role in maturation in that the external mould layer provides a kind of defence against the settling down of harmful microbes and against too quick drying.

On considering the results of our experiments, the question of a controlled development of the mould cover automatically arises. The use of pure cultures has been introduced into almost all branches of food industry connected with microbiology. Application of pure cultures in the manufacture of Hungarian

salami seems desirable just as it is already indispensable in the distilling industry, in yeast production, in the brewery, in butter-making, in cheese manufacture, in butanol fermentation, etc.

According to present practice, formation of the mould cover is left to «chance», *i. e.* on the qualitative and quantitative composition of the microflora

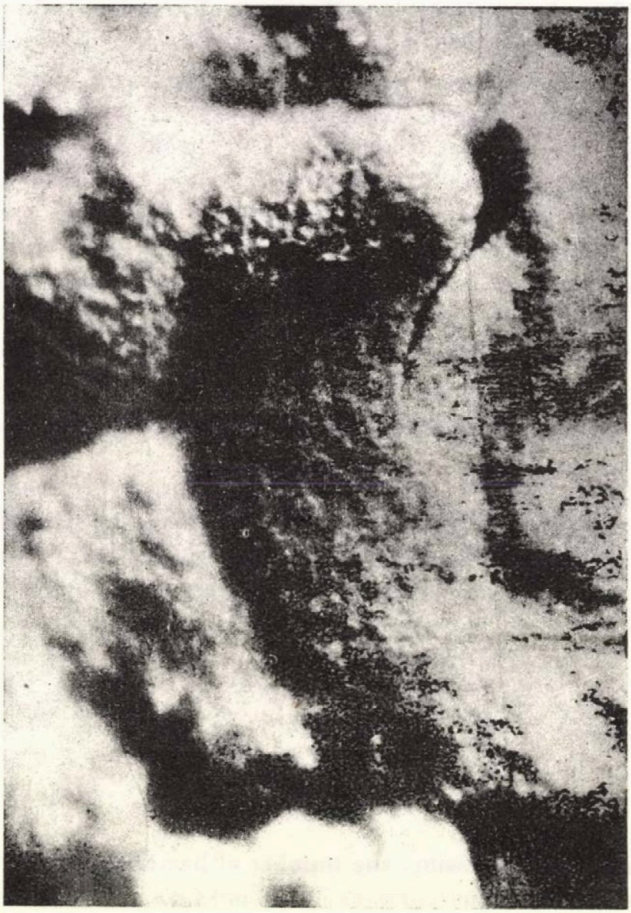

Fig. 4. Cover formed by *Scopulariopsis brevicaulis* on the surface of salami (magnification about 50 fold)

in the air actually prevailing in the drying room. Thus, development of the mould layer begins in patches and is completed slowly. The outward appearance of the cover, being a function of the microflora present in the surrounding air, is not invariably uniform.

All these difficulties may be eliminated by bathing or spraying the sausages, before drying, with a suspension of the conidia of a mould chosen pre-

viously for its ability to form layers of suitable colour and texture. Suspensions prepared in sterile, 0,2 per cent agar solutions and containing 10^7 to 10^9 conidia per ml proved adequate to provide a massive infection.

Only a few of our experiments on controlled cover-formation will be mentioned here.

Using spore suspensions prepared by mass-propagation of *Aspergillus candidus* and *Scopulariopsis brevicaulis*, originally isolated from salami, the mould layer could *completely* be developed in 2 or 3 weeks, whilst with the present «spontaneous» method this process took about the same number of months. The difference may be illustrated by Fig. 2.

Concerning the quality of the cover, it should be noted that this, of course, has to be fixed by taking account of certain technological aspects and of the taste of the consumers. In the case of the above two species of fungi, it can be stated that the layer produced by the *Aspergillus* is snow-white and rather loose in structure while the cover formed by *Scopulariopsis* is of a yellowish-, greyish-white colour and of compact texture. Microscopic examination of the respective salami surfaces clearly shows this difference (Figs. 3. and 4.).

As a result of the above characteristics, protecting covers formed by *Aspergillus* are more easily damaged mechanically than those produced by *Scopulariopsis*.

By using pure cultures, results in salami manufacture may be improved. Before practical application, thorough research has to be carried out into the cover-forming properties, the colour, texture, and mechanical characteristics of many strains of mould. Consumer preference has to be analysed statistically. With this information at our disposal, the outward appearance of salami may be controlled and standardised at will.

Acknowledgement. The invaluable help of F. KOVÁCS (Salami Factory, Budapest) is greatfully acknowledged.

Summary

In the course of processing, the number of bacteria (the chief constituents of the microflora of the inner portions of salami) first increases. During ripening the bacterial count stagnates for a while and then, from the 50th, or 60th day onwards, slowly decreases.

Initially, after smoking, the microflora of the surfaces of Hungarian salami consists mainly of yeast-like organisms and bacteria which, later on, are overgrown by various *Penicillium*, *Scopulariopsis* and *Aspergillus* species. The ratio of these microbes and the date of their appearance is a function of the microflora, the temperature and relative humidity of the air in the drying room. The effect on flavour development of the external mould cover proved to be indirect; it manifests itself not in the production of flavour substances

but in preventing the development of undesirable micro-organisms and in hindering rapid, uneven drying.

The development of the external microflora can be controlled by applying suspensions of conidia from pure cultures. By this method the mould cover can be developed more quickly and uniformly and in a quality complying with industrial requirements.

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ЗАМЕЧАНИЯ О МИКРОФЛОРЕ САЛЯМИ И ЕЕ НАПРАВЛЕННОМ ОБРАЗОВАНИИ

К. ВАШ и Г. ПУЛАИ

Резюме

Число бактерий, образующих микрофлору внутренней части салями, в процессе изготовления сперва повышается. В периоде созревания (сушения), в течение некоторого времени остается без изменения, затем, приблизительно по истечении 50–60 дней, начинает медленно понижаться.

Микрофлора наружности венгерского салями вначале (непосредственно после копченья) состоит главным образом из дрожжеподобных микробов и бактерий, позже преобладают различные виды **Penicillium, Scopulariopsis** и **Aspergillus.** Числовое отношение и время появления этих микробов зависит от воздуха сушильного помещения: его микрофлоры, температуры и содержания влаги. Согласно проведенным исследованиям, роль внешней микрофлоры венгерского салями на образование вкуса вероятно носит только посредственный характер: выявляется не в образовании вкусовых веществ, а в недопускании вредных микроорганизмов и в предохранении от быстрого неравномерного высыхания.

Образование внешней микрофлоры может происходить направленно с помощью суспензии конидий чистых культур плесневых грибков. Этим методом плесневую оболочку на поверхности салями можно образовать быстрее, равномернее и в соответствующем требованиям промышленности качестве.

DER ERREGER DER VIRUSKRANKHEIT VON HYPHANTRIA CUNEA DRURY

Von

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Der amerikanische weisse Spinner, *Hyphantria cunea Drury*, hat sich in Ungarn 1940 angesiedelt. Im Laufe von sieben Jahren hat er sich über die Hälfte des Landes verbreitet und war bereits 1948 im ganzen Land als einer der gefährlichsten Schädlinge zu betrachten. Die rasche Verbreitung ist wahrscheinlich darauf zurückzuführen, dass in der neuen Heimat natürliche Feinde und Krankheiten verursachende Mikroorganismen nicht vorhanden sind. In Nordamerika wird dieser Spinner von mehreren Insektenparasiten sowie Mikroorganismen angegriffen [1], so dass er kaum merkbaren Schaden anrichtet.

Mit verschiedenen chemischen und mechanischen Methoden ist in Ungarn versucht worden, seine Vermehrung zu verhindern, doch war der Erfolg bisher gering. Da wir auch an die in Ungarn einheimischen Insektenparasiten keine grossen Hoffnungen knüpfen dürfen [2], sehen wir die Möglichkeit eines erfolgreichen Schutzes nur in mikrobiologischen Methoden. Für diesen Zweck sind vor allem Protozoen und Viren geeignet.

Nachdem bereits eine Methode ausgearbeitet worden war, die als Krankheitserreger ein Protozoon zur Anwendung brachte [6], berechtigt die Tatsache zu neuen Hoffnungen, dass 1953 im Insektarium Raupen gefunden wurden, welche die charakteristischen Symptome einer Virusinfektion aufwiesen. 1954 konnten wir auch im Freien Raupennester beobachten, in denen die Larven an Polyeder-Infektion litten. In deren Körper waren auch die charakteristischen Einschlusskörperchen in grosser Zahl anwesend, so dass die Möglichkeit bestand, diese im Elektronenmikroskop zu untersuchen. Der eigentliche Krankheitserreger befindet sich im Inneren dieser Einschlusskörperchen. Unter natürlichen Verhältnissen erfolgt die Infektion durch den Mund, und die Einschlusskörperchen geben ihren Virusgehalt auf Wirkung der alkalischen Darmsäfte der Raupen ab. Wir reproduzierten diesen Prozess in vitro und wandten bei diesem Verfahren folgende Methode an:

Präparatives Verfahren

Nachdem die Einschlusskörperchen von Zellfragmenten, Gewebselementen und Fett durch Filtration und wiederholtes Zentrifugieren gereinigt wurden,

behandelten wir die Suspensionen von den bisherigen Methoden abweichend [1, 4, 9, 10, 11] folgendermassen:

1. Zur Durchführung der in einer früheren Arbeit [7] mitgeteilten Tropfendialyse fertigten wir nach der KELLENBERGERschen Methode [5] auf der Oberfläche einer Agarplatte eine Kollodiummembran an. Von dieser wurde ein der Grösse eines Objektträgers entsprechender Teil herausgeschnitten und auf die Oberfläche von n/10 NaOH-Lösung übertragen.

Auf die auf der Lösung schwimmende Membran wurden nunmehr winzige Tropfen der Einschlusskörperchen-Suspension übertragen und dem Prozess der Dialyse überlassen. Nach verschiedenen Inkubationszeiten entfernten wir die Membran von der Oberfläche der Lösung mit einem von einer Agarplatte bedeckten Objektträger und übertrugen sie auf destilliertes Wasser. Das destillierte Wasser entfernte aus den auf der Oberfläche der schwimmenden Membran befindlichen Tropfen — ebenfalls durch Dialyse — auch die letzten Spuren der Lauge. Endlich wurden die Membranen auf die vorhin angeführte Weise von der Wasseroberfläche abgehoben, fixiert, in kleine Stückchen zerlegt und sodann diese Stückchen am Objektträger befestigt nach Palladiumbeschattung untersucht.

Dieses Verfahren ermöglichte die schonende Behandlung der Einschlusskörperchen, und wir halten es — auch auf Grund unserer früheren, mit dem *Bombyx mori*-Virus durchgeführten Untersuchungen — für wahrscheinlich, dass es neben der Ausschaltung der Selektivität auch die topographischen Verhältnisse des Lösungsprozesses getreu bewahrt.

2. Da es bei der angeführten Methode trotzdem vorkam, dass Teile der gelösten Einschlusskörperchen von ihrem ursprünglichen Platz in grössere Entfernung abwanderten, als es im Interesse der genauen Beobachtung zulässig war, suchten wir das Verfahren zu verbessern. Zu diesem Zweck brachten wir die in Wasser suspendierten Polyederkörperchen in Sprayform auf die nach KELLENBERGER bereitete Kollodiummembran und liessen sie eintrocknen. Die in kleine Würfel geschnittenen Membranteile übertrugen wir hiernach auf die Oberfläche der n/10 NaOH-Lösung und dialysierten sie hier. Die trockenen Polyederkörperchen saugten durch die Membran gerade soviel Lösung in sich auf, als zu ihrer Auflösung erforderlich war. Nach verschiedenen Inkubationszeiten entfernten wir die Präparate von der Lauge und behandelten sie nach der beim vorigen Verfahren beschriebenen Methode weiter. Bei diesem Verfahren nehmen an der Lösung nur ganz geringe Flüssigkeitsmengen teil. Infolgedessen bleiben die gelösten Teile auf einem scharf begrenzten Gebiet, wodurch die genaueste Beobachtung des Prozesses und Bestimmung des Virusgehaltes ermöglicht wird.

Wir untersuchten die Präparate im TTC-Elektronenmikroskop bei 40 kV-Spannung.

Ergebnisse

Die von den im Winkel von 45° beschatteten intakten Polyedern hergestellten Aufnahmen zeigen, dass die ungelösten Einschlusskörperchen würfelförmig sind mit abgeflachten Kanten. Sie sind kleiner als die der Polyeder der Seidenraupe, ihr Durchmesser beträgt 2—3 Mikron (Abb. 1).

Dialysieren wir die Polyeder, können wir leicht feststellen, dass sie — obwohl die Einschlusskörperchen bei Lösung nach den oben beschriebenen Behandlungsmethoden ein charakteristisch verschiedenes Bild aufweisen —

Abb. 1. Hyphantria-Polyederkörperchen, nach negativ-Beschattung im Winkel von 45°

in der Eigenschaft übereinstimmen, dass sie gegenüber der Lösungwirkung der Lauge verschiedenen Widerstand leisten. So können wir in ein und demselben Gesichtsfeld in verschiedenem Lösungszustand befindliche Einschlusskörperchen sehen (Abb. 2). Benutzen wir die Tropfendialyse, bleibt die die äussere Hülle der Polyeder bildende Membran beinahe unversehrt. Zu Beginn der Auflösung entfernen sich aus dem Inneren des Polyeders wolkenartige Flocken, welche die Proteine mit niedrigem Molekulargewicht des Einschlusskörperchens repräsentieren. Die äussere Membran tritt in scharfen Umrissen in Erscheinung und entleert sich — parallel mit der allmählichen Auflösung des kleinmolekulären Proteingehaltes — mehr und mehr. Bald sind im Inneren der Membran auch Virusbündel zu erblicken, die bisher vom kleinmolekulären Proteingehalt verdeckt waren. In diesem Stadium sind auch schon einzelne individuelle Virusstäbchen zu beobachten. Die äussere Membran löst sich nicht auf, sondern behält ihren filmartigen Zustand bis zur Beendigung der Auflösung (Abb. 3).

Bei der Trockendialyse werden die gelösten Bestandteile von den Flüssigkeitsströmungen kaum von ihrer Stelle bewegt. Die ganze Substanz, die sich bei der Dialyse nicht durch die Kollodiummembran entfernen konnte, bleibt

an ihrer Stelle, und wir gewinnen ein charakteristisches Bild: an der reinen Membran sehen wir rundliche Granula von etwa 10—20 Millimikron Durchmesser, die teils aus dem kleinmolekulären Protein, teils aus der sich ebenfalls auflösenden äusseren Membran stammen. Auf dieser Basis liegen scharf begrenzte Virusbündel und individuelle Virusstäbchen (Abb. 4).

Auffallend war, dass die Einschlusskörperchen des *Hyphantria cunea Drury* im Gegensatz zu den Polyedern des Bombyx mori lediglich 10—20 Virusbündel enthielten. Die Bündel haben eine Grösse von 400×200 bzw. 400×300

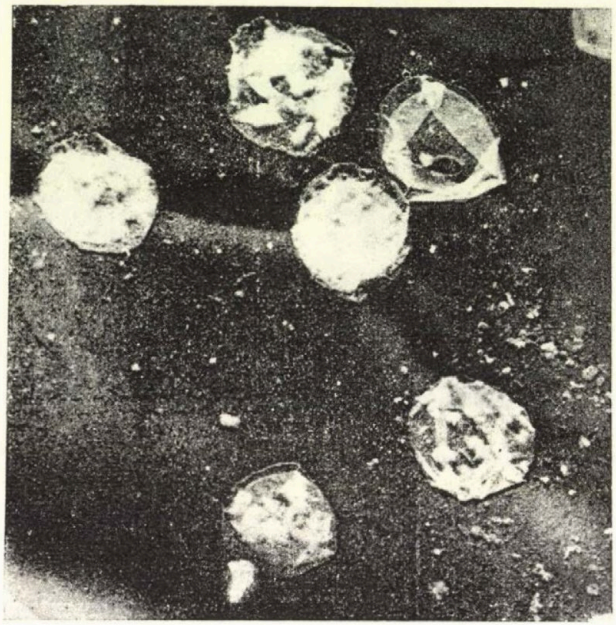

Abb. 2. Mit Tropfendialyse behandelte Polyeder in verschiedenen Stufen der Auflösung

Millimikron. Es ist gut zu sehen, dass auch diese Bündel von einer Membran oder Kapsel eingehüllt sind und dass aus ihnen bei fortschreitender Auflösung 3—6 Virusstäbchen frei werden (Abb. 5).

Die individuellen Virusstäbchen, die nach Trockendialyse zu sehen sind, haben eine Grösse von 330×50 Millimikron und sind an den Enden abgerundet. Bei der Tropfendialyse hingegen beträgt ihre Breite, obwohl die Länge der Stäbchen mit der der vorigen übereinstimmt, 90—100 Millimikron. Auch in der Form weisen sie Abweichungen auf: die Enden sind nicht abgerundet, sondern gerade abgeschnitten, im Inneren ist eine parallele Struktur zu sehen. Stäbchen von ähnlicher Form sind wir bereits 1954 beim Bombyx mori-Virus begegnet, doch zeigten diese Stäbchen an ihren Enden eine Verdickung. Wir

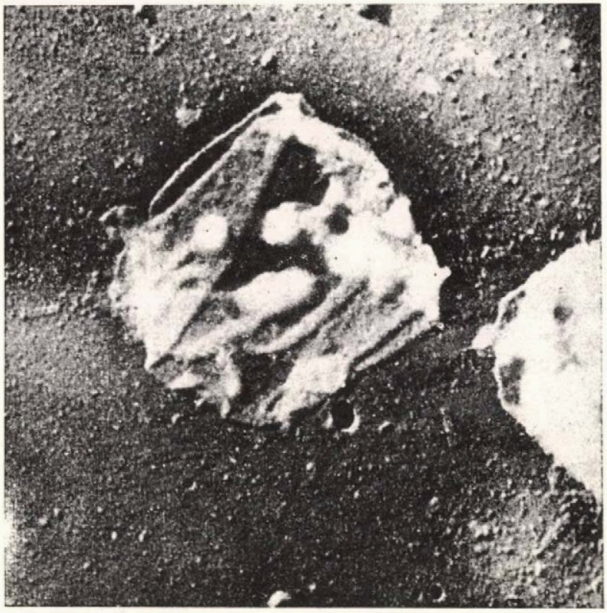

Abb. 3. In Auflösung befindliches Einschlusskörperchen und Elementarstäbchen nach Behandlung mit Tropfendialyse

Abb. 4. Zwischen der äusseren Membran und den Resten des aufgelösten kleinmolekulären Polyeder-Proteins sichtbare Virusbündel und Elementarstäbchen, nach Trockendialyse

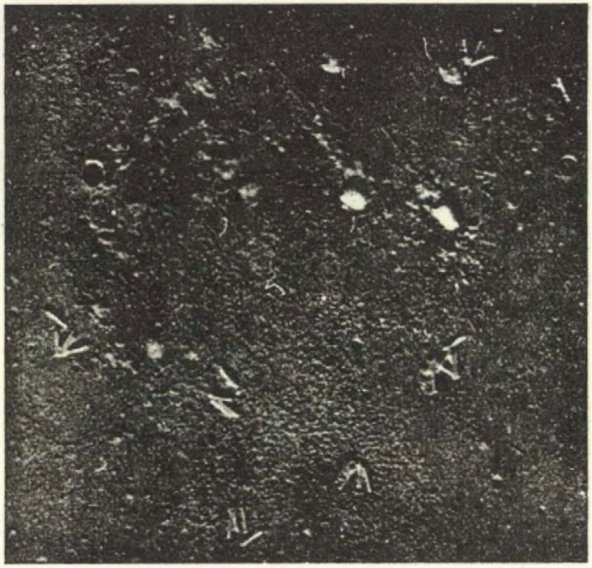

Abb. 5. Aus Virusbündeln freiwerdende Elementarstäbchen nach Trockendialyse

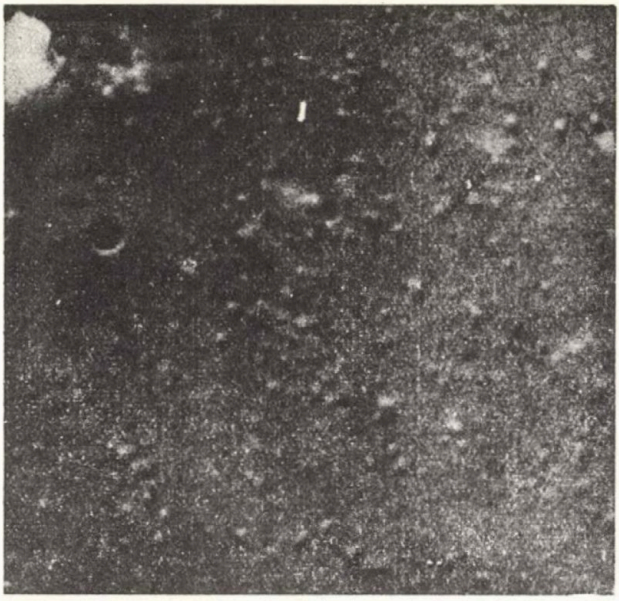

Abb. 6. Elementarstäbchen, wie sie nach Tropfendialyse zu beobachten sind

nehmen an, dass die individuellen Stäbchen im Inneren der Virusbündel zu zweien liegen, und zwar in einer weiteren Kapsel, der Periviruskapsel, eingeschlossen, und dass diese innerste Hülle die elementaren Körperchen erst auf die starke Einwirkung der Lauge hin freilässt.

Vergleichen wir das Hyphantria-Virus mit den Viren anderer europäischer Schmetterlinge, so sehen wir, dass dieses den Viren des *Dendrolimus pini* Linn. und *Callimorpha dominula* Linn. ähnelt. Wir halten es für möglich, dass sich einzelne Stämme der Viren der alten einheimischen Arten an den amerikanischen weissen Spinner, der sein ursprüngliches Virus in seiner Heimat zurückliess, gewöhnt haben. Es ist zu hoffen, dass das gefundene Virus im Rahmen der mikrobiologischen Abwehr zur Bekämpfung des Schädlings wertvolle Hilfe bieten wird.

Zusammenfassung

1954 wurden *Hyphantria cunea Drury*-Nester beobachtet, in denen die Larven an Polyedervirusinfektion erkrankt waren. Die für diese Krankheit charakteristischen Einschlusskörperchen wurden elektronenmikroskopisch untersucht. Hierfür sind zwei Verfahren ausgearbeitet worden : die zu untersuchenden Polyeder wurden in Wasser suspendiert bzw. im trockenen Zustand auf einer auf n/10 NaOH-Lösung schwimmenden Kollodiummembran gegen Lauge dialysiert. Die Einschlusskörperchen lösten sich durch die Wirkung der Lauge auf. Nachdem die Lauge durch Dialyse gegen destilliertes Wasser aus dem Präparat entfernt worden war, wurde die selektive Wirkung der Behandlung aufgehoben, so dass der Auflösungsprozess mit der grössten topographischen Genauigkeit beobachtet werden konnte.

Die Einschlusskörperchen sind 2—3 Mikron gross und würfelförmig. Im Laufe der Auflösung entleeren sich aus ihnen zuerst kleinmolekuläre Proteine. Später werden 10—20 Stück 400×300 oder 400×200 Millimikron grosse Virusbündel sichtbar, die von einer Membran bedeckt sind. In ihrem Inneren befinden sich 3—6 Virusstäbchen. Diese sind nach Trockendialyse 330×50, nach Tropfendialyse 330×100 Millimikron gross. Die trockene Dialyse repräsentiert ein späteres Stadium der Auflösung, die breiteren Formen enthalten zwei nebeneinander liegende Einzelkörperchen, die parallel in eine Periviruskapsel eingebettet sind.

Da der amerikanische weisse Spinner bisher in Ungarn ohne ernsthafte Parasiten und Krankheitserreger lebte, ist zu hoffen, dass es gelingen wird, seiner Ausnahmestellung mit Hilfe des beschriebenen Virus ein Ende zu bereiten, und dass das Virus auch bei der biologischen Ausrottung eine wertvolle Hilfe bieten wird.

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НОВОВЫДЕЛЕННЫЙ ВИРУС АМЕРИКАНСКОГО БЕЛОГО ШЕЛКОПРЯДА

Л. МАХАИ и Б. ЛОВАШ

Резюме

В 1954 году авторы наблюдали гнезда бабочек **Hyphantria cunea Drury**, личинки которых были поражены полиэдровым вирусом. Характерные для этого заболевания включения подвергались электронмикроскопическому исследованию. С этой целью разработали два способа: подлежащие исследованию полиэдры, взвешенные в воде, или в сухом состоянии на пленке коллодия, плавающей на поверхности раствора $n/^{10}$ NaOH, диализировали напротив щелочи. Под действием щелочи включения растворялись. После удаления щелочи также путем диализа против дестилированной воды, устранилось селективное действие обработки, так что процесс растворения мог наблюдаться с наибольшей топографической точностью. Включения имеют форму гексаэдров, размера 2—3 μ. В процессе растворения вначале удаляются маломолекулярные протеины, позже обнаруживаются 10 20 пучков вирусов, покрытых оболочкой, размером 400×300 или 400×200 mμ. Внутри них располагается по 3 6 вирусных палочек. Размеры их после сухого диализа 330×50 mμ, а после капельного диализа 330×100 mμ. Сухой диализ представляет более позднюю стадию растворения, более широкие формы состоят из двух элементарных телец, расположенных параллельно друг другу и заключенных в оболовирусную капсулу.

Так как до настоящего времени американский белый шелкопряд существовал в нашей стране без значительных паразитов и возбудителей, авторы надеются, что с помощью описанного вируса удастся прекратить его исключительное положение и это окажет ценную помощь при биологическом истреблении данных насекомых.

IMMUNOLOGICAL RELATION OF DONKEY, MULE AND HORSE SERUM ALBUMIN

By

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Numerous authors have studied the immunology of serum albumins. According to TAYLOR, ADAIR and ADAIR [1], crystalline horse serum albumin behaves as a complex antigen. GOLDSWORTHY and RUDD [2] have shown that the cause of this apparent complexity is to be sought in the contamination of albumin preparations with globulin. KABAT and HEIDELBERGER [3] studied the reaction between globulin-free horse serum albumin and rabbit anti-albumin in quantitative precipitation tests and showed that the system was homogeneous.

Concerning the serological relations between serum albumins of different species, literature contains little information. According to ADAIR and HAMILTON [4], the antisera produced in the rabbit against crystalline human albumin and against horse serum albumin, give a cross reaction in test with the heterologous albumin in case prolonged immunization. In the quantitative tests made by MELCHER, MASOUREDIS and REED [5] the antibodies produced against human or bovine serum albumin gave a cross reaction of about 15 per cent.

In the study to be reported crystalline serum albumins from three closely related species (donkey, mule and horse), were examined for immunological relationship, using semiquantitative and quantitative techniques.

Materials and methods

Preparation of serum albumins. Crystalline serum albumins were prepared according to MCMEEKIN [6]. The preparations of serum albumin were stored in the form of 1 to 4 per cent solutions in the frozen state until use.

Production of antialbumins. Rabbits ranging in weight from 3000 g to 4000 g were immunized intravenously, at 3 day intervals with aliquot volumes of serum albumin which had been crystallized four times, dialysed, sterilized by filtration and preserved with 0,01 per cent Merthiolate. During the six weeks of the immunization cycle the total dose for each rabbit was 650 mg of donkey albumin, or 600 mg of mule or 600 mg of horse serum albumin. Seven days after the last dose of antigen had been administered the rabbit was killed by bleeding under sterile conditions. The mixture of sera was sterilized by filtration, preserved with 0,01 per cent Merthiolate, and stored in the frozen state.

Precipitation tests. The reaction between antisera and homologous albumin and heterologous albumins, respectively, was studied by semi-quantitative techniques (CULBERTSON's [7] neutralization test) determination of optimum points (DEAN and WEBB [8]), as well as by the quantitative precipitation method introduced by HEIDELBERGER and KENDALL [9], as modified by GITLIN [10], using a Beckman spectrophotometer.

Results

In the first part of the experiments the serological reaction between the albumins and the antialbumins produced against them was studied by semiquantitative techniques. To fourfold dilutions of antisera were added various amounts of the heterologous or homologous albumins (100 to 1,0 μg N), the test volumes having been 0,5 ml each. After 2 hours at 37° C, then 24 hours at 4° C, the precipitates were centrifuged and the supernatants divided into four equal parts. Then to each of the portions of the supernatant were added 0,2 ml of the 1 : 10 dilution of the test serum, or 40 μg doses of the three crystalline albumins, in 0,2 ml volume. It was thus examined whether we could find in our systems a neutralization point or zone in which no excess of antigen or of antibody is present in the supernatant. In other words, we wanted to determine whether the crystalline serum albumins tested were immunologically homogeneous or complex. In the case of complex antigens, analysis of the supernatants will yield a wide zone, within which both antigen and antibody are demonstrable. At the same time we wanted also to determine in what measure homologous absorption of antialbumins was capable of eliminating from the serum the antibodies reacting with heterologous albumins, and *vice versa*, in order to find out whether the cross reactions were due to the presence of common part-antigens or to an identity of the antigens.

In the above tests the crystalline donkey, mule and horse serum albumins proved to be homogeneous antigens. Analysis of the supernatants further showed that absorption of any antialbumin with any of our albumins resulted in a simultaneous elimination from the serum of all antibody capable of reacting with the other two types of albumin. Thus, the three kinds of albumin tested proved to be antigens identical as what regards immuno-specificity.

The position of the neutralization points or of zones, as determined by the above method, as well as the determination of the constant antibody-optimum points of DEAN and WEBB (the tubes showing the fastest precipitation after various amounts of antigen had been added to equal amounts of serum), permitted also of quantitative conclusions. So, for example, the quantity of antibody in mule antialbumin was approximately 50 per cent of that contained in either donkey, or horse antialbumin. It was also found that the single types of serum albumin differ in their ability to bind antibody. In order thoroughly to check these findings, the homologous and the heterologous systems were examined also by a quantitative precipitation method.

In possession of the data of semiquantitative analysis, quantitative precipitation was carried out with 1 : 4 dilutions of donkey and horse, and 1 : 2 dilutions of mule antialbumins, using 0,5 ml of each in the tests against 0,005 to 0,100 mg antigen N (also in 0,5 ml volume). After standing 2 hours at 37° C and 24 hours at 4° C the precipitates formed were centrifuged off and washed

Table I

Precipitation test with donkey antialbumin

(0,5 ml. 1 : 4 dil. serum)

Tube No.	Ant—N added mg.	Ant—N pptd.	Total—N pptd. mg.	Ab—N pptd by difference mg.	Ratio Ab—N : Ant—N in ppt.	Ab—N pptd calculated by equation 1.	Ab—N pptd calculated by equation 2.	Tests on supernatant.
				A) + Donkey Albumin				
1.	0,010	total	0,094	0,084	8,4	0,084	0,093	Excess Ab.
2.	0,015	«	0,131	0,116	7,7	0,115	0,121	« «
3.	0,020	«	0,170	0,150	7,2	0,137	0,140	« «
4.	0,025	«	0,179	0,152	6,1	0,152	0,153	∅
5.	0,030	near total	0,198	0,168	5,6	0,159	0,158	Traces Ant.
6.	0,035	partial	0,200	0,165	4,7	0,159	0,158	Little «
7.	0,040	«	0,200	0,160	4,0	0,151	0,152	« «
8.	0,050	«	0,187					Excess «
9.	0,070	«	0,158					« «
10.	0,100	«	0,134					« «

Equation 1. Ab—N ppt $= 9{,}9\ x - 153\ x^2$; Ant. max.: 0,032, Ab. max.: 0,160
Equation 2. Ab—N ppt $= 14{,}8\ x - 55\ x^{3/2}$; Ant. max.: 0,032, Ab. max.: 0,159

				B) + Mule albumin				
1.	0,010	total	0,117	0,107	10,7	0,081	0,092	Excess Ab.
2.	0,015	«	0,134	0,119	7,9	0,112	0,122	« «
3.	0,020	«	0,157	0,137	6,8	0,138	0,146	« «
4.	0,025	«	0,171	0,146	5,8	0,158	0,163	∅
5.	0,035	near total	0,205	0,170	4,9	0,181	0,182	Traces Ant.
6.	0,040	partial	0,224	0,184	4,6	0,184	0,184	Little Ant.
7.	0,045	«	0,230	0,185	4,1	0,182	0,182	« «
8.	0,055	«	0,189					Excess Ant.
9.	0,070	«	0,157					« «
10.	0,100	«	0,141					« «

Equation 1. Ab—N ppt $= 9{,}2\ x - 115\ x^2$; Ant. max.: 0,040 Ab. max.: 0,184
Equation 2. Ab—N ppt $= 13{,}8\ x - 46\ x^{3/2}$; Ant. max.: 0,040 Ab. max.: 0,184

				C) + Horse Albumin				
1.	0,010	total	0,066	0,056	5,6	0,056	0,059	Excess Ab.
2.	0,015	«	0,085	0,070	4,7	0,071	0,080	« «
3.	0,020	«	0,106	0,186	4,3	0,089	0,097	« «
4.	0,030	«	0,141	0,111	3,7	0,117	0,122	« «
5.	0,040	«	0,176	0,136	3,4	0,135	0,136	« «
6.	0,045	«	0,184	0,139	3,1	0,141	0,139	∅
7.	0,055	near total	0,197	0,142	2,6	0,143	0,139	Traces Ant.
8.	0,060	partial	0,205	0,145	2,4	0,140	0,136	Little Ant.
9.	0,080	«	0,201					Excess Ant.
10.	0,100	«	0,208					« «

Equation 1. Ab—N ppt $= 5{,}5\ x - 53\ x^2$; Ant. max.: 0,052 Ab. max.: 0,143
Equation 2. Ab—N ppt $= 8{,}4\ x - 25\ x^{3/2}$; Ant. max.: 0,050 Ab. max.: 0,140

Table II

Precipitation test with mule antialbumin

(0,5 ml. 1 : 2 dil. serum)

Tube No.	Ant—N added mg.	Ant—N pptd.	Total—N pptd. mg.	Ab—N pptd by difference mg.	Ratio Ab—N : Ant—N in ppt.	Ab—N pptd calculated by equation 1.	Ab—N pptd calculated by equation 2.	Tests on supernatant.
				A) + Donkey albumin				
1.	0,005	total	0,067	0,062	12,4	0,063	0,081	Excess Ab.
2.	0,010	«	0,120	0,110	11,0	0,110	0,119	« «
3.	0,020	«	0,170	0,150	7,2	0,164	0,165	∅
4.	0,025	near total	0,192	0,167	6,7	0,167	0,170	Traces Ant.
5.	0,030	partial	0,195	0,165	5,5	0,160	0,163	Little Ant.
6.	0,035	«	0,202	0,167	4,8	0,137	0,149	« «
7.	0,045	«	0,195					Excess Ant.
8.	0,055	«	0,165					« «
9.	0,080	«	0,144					« «
10.	0,100	«	0,120					« «
	Equation 1. Ab—N ppt = 13,9 x — 286 x^2				Ant. max.: 0,024		Ab. max.: 0,169	
	Equation 2. Ab—N ppt = 20,7 x — 88 $x^{3/2}$				Ant. max.: 0,025		Ab. max.: 0,170	
				B) + Mule albumin				
1.	0,005	total	0,069	0,064	12,8	0,076	0,099	Excess Ab.
2.	0,010	«	0,141	0,131	13,1	0,131	0,139	« «
3.	0,015	«	0,179	0,164	10,9	0,165	0,169	∅
4.	0,020	near total	0,195	0,175	8,8	0,179	0,179	Traces Ant.
5.	0,025	partial	0,198	0,173	6,9	0,172	0,175	Little Ant.
6.	0,030	«	0,203	0,173	5,8	0,144	0,155	« «
7.	0,040	«	0,141					Excess Ant.
8.	0,060	«	0,131					« «
9.	0,080	«	0,123					« «
10.	0,100	«	0,104					« «
	Equation 1. Ab—N ppt = 17,2 x — 413 x^2				Ant. max.: 0,021		Ab. max.: 0,179	
	Equation 2. Ab—N ppt = 25,8 x — 119 $x^{3/2}$				Ant. max.: 0,021		Ab. max.: 0,179	
				C) + Horse albumin				
1.	0,005	total	0,059	0,054	10,8	0,043	0,057	Excess Ab.
2.	0,010	«	0,091	0,081	8,1	0,081	0,093	« «
3.	0,015	«	0,131	0,116	7,7	0,114	0,126	« «
4.	0,025	«	0,171	0,146	5,9	0,166	0,172	« «
5.	0,035	«	0,212	0,177	5,1	0,196	0,198	∅
6.	0,045	near total	0,251	0,206	4,6	0,206	0,207	Traces Ant.
7.	0,050	partial	0,254	0,204	4,1	0,205	0,205	Little Ant.
8.	0,055	«	0,253	0,198	3,6	0,198	0,201	« «
9.	0,070	«	0,261					Excess Ant.
10.	0,100	«	0,235					« «
	Equation 1. Ab—N ppt = 9,1 x — 100 x^2				Ant. max.: 0,046		Ab. max.: 0,207	
	Equation 2. Ab—N ppt = 13,5 x — 42 $x^{3/2}$				Ant. max.: 0,046		Ab. max.: 0,207	

Table III

Precipitation test with horse antialbumin

(0,5 ml. 1 : 4 dil. serum)

Tube No.	Ant—N added mg.	Ant—N pptd.	Total—N pptd. mg.	Ab—N pptd by difference mg.	Ratio Ab—N : Ant—N in ppt.	Ab—N pptd calculated by equation 1.	Ab—N pptd calculated by equation 2.	Tests on supernatant.
				A) + Donkey albumin				
1.	0,005	total	0,114	0,109	21,9	0,077	0,101	Excess Ab.
2.	0,010	«	0,158	0,148	14,8	0,132	0,140	« «
3.	0,105	«	0,160	0,145	9,7	0,166	0,168	∅
4.	0,020	near total	0,197	0,177	8,8	0,177	0,177	Traces Ant.
5.	0,025	partial	0,200	0,175	7,0	0,167	0,170	Little Ant.
6.	0,035	«	0,213					Excess Ant.
7.	0,050	«	0,179					« «
8.	0,070	«	0,144					« «
9.	0,100	«	0,104					« «
10.								
	Equation 1. Ab—N ppt = 17,6 x — 437,5 x^2				Ant. max.: 0,020		Ab. max.: 0,177	
	Equation 2. Ab—N ppt = 26,4 x — 124 $x^{3/2}$				Ant. max.: 0,020		Ab. max.: 0,177	
				B) + Mule albumin				
1.	0,005	total	0,086	0,081	16,2	0,048	0,063	Excess Ab.
2.	0,010	«	0,132	0,122	12,2	0,084	0,096	« «
3.	0,015	«	0,154	0,139	9,3	0,118	0,124	« «
4.	0,020	«	0,168	0,148	7,4	0,140	0,142	∅
5.	0,030	near total	0,187	0,157	5,2	0,157	0,157	Traces Ant.
6.	0,035	partial	0,181	0,146	4,2	0,154	0,153	Little Ant.
7.	0,050	«	0,165					Excess Ant.
8.	0,070	«	0,150					« «
9.	0,100	«	0,104					« «
10.								
	Equation 1. Ab—N ppt = 10,4 x — 172 x^2				Ant. max.: 0,030		Ab. max.: 0,157	
	Equation 2. Ab—N ppt = 15,6 x — 60 $x^{3/2}$				Ant. max.: 0,030		Ab. max.: 0,157	
				C) + Horse albumin				
1.	0,010	total	0,088	0,078	7,8	0,073	0,082	Excess Ab.
2.	0,015	«	0,123	0,108	7,2	0,100	0,108	« «
3.	0,020	«	0,141	0,121	6,1	0,120	0,125	« «
4.	0,025	«	0,160	0,135	5,4	0,136	0,137	∅
5.	0,035	near total	0,178	0,143	4,1	0,144	0,144	Traces Ant.
6.	0,045	partial	0,194	0,149	3,3	0,127	0,132	Little Ant.
7.	0,055	«	0,200					Excess Ant.
8.	0,080	«	0,190					« «
9.	0,100	«	0,162					« «
10.								
	Equation 1. Ab—N ppt = 8,5 x — 126 x^2				Ant. max.: 0,034		Ab. max.: 0,144	
	Equation 2. Ab—N ppt = 12,9 x — 47 $x^{3/2}$				Ant. max.: 0,034		Ab. max.: 0,144	

three times with chilled physiological saline. Subsequently, the precipitates were dissolved in 1,5 ml of 0,1 N acetic acid. Extinctions were read at 279 mμ, after 3 hours at room temperature.

The results are shown in Tables I. to III.

For characterising different precipitation systems (in the zone of antibody excess, untill the zone of equivalence) the following types of empirical equations are used [11].

1. Antibody N in precipitate $= ax - bx^2$, or
2. Antibody N in precipitate $= cx - dx^{3/2}$, where

x = precipitated (added) antigen N,

a, b, c and d = constants calculated from experimental data (corresponding antibody N and antigen N values).

The above equations can be expressed also as follows.

1. Antibody N in precipitate $= 2Rx - \frac{R^2}{A} x^2$, or
2. Antibody N in precipitate $= 3Rx - 2\sqrt{\frac{R^3 x^3}{A}}$, where

x = precipitated (added) antigen N,

A = antibody N precipitated at some end point (dependent on the system studied) of the zone of equivalence,

R = the ratio of precipitated antibody N to precipitated antigen N at the reference point.

A comparison of the above equations reveals that

$$a = 2r$$

$$b = \frac{R^2}{A}$$

$$c = 3R$$

$$d = 2\sqrt{\frac{R^3}{A}}$$

Thus, the values may be calculated according to either equation, but may deviate in actual value, depending on the specific properties of the systems examined and on the limits or error of the estimation.

In Tables I to III are shown the equations for the single systems ; the data on the actually determined composition of precipitates for different concentrations of antigen ; the appropriate antibody N values calculated from the equations ; and also the results of supernatant analyses. Also included are the maximum antibody N and the appropriate maximum antigen N values, as calculated by differentiating of the corresponding equations.

According to the data in Tables I to III (as it could be anticipated on grounds of the results of semiquantitative tests), certain quantitative differences in behaviour are detectable between the single systems. In order to facilitate a better comparison of these differences, in Table IV we have shown the maximum antibody-N contents of the different antisera, as well as their appropriate maximum antigen-N values.

Table IV

Summarized results of quantitative precipitation tests

Antigen used for absorption	Determined values			Calculated values					
				Calculated by equation 1.			Calculated by equation 2.		
	Ant—N max. mg.	Ab—N max. mg.	Ab—N max. mean. mg.	Ant—N max. mg.	Ab—N max. mg.	Ab—N max. mean. mg.	Ant—N max. mg.	Ab—N max. mg.	Ab—N max. mean. mg.
I. Donkey antialbumin (0,5 ml, 1 : 4 dil.)									
Donkey alb....	0,030	0,168		0,032	0,160		0,032	0,159	
Mule « ...	0,045	0,185	0,165	0,040	0,184	0,164	0,040	0,184	0,164
Horse « ...	0,060	0,145	±12,1%	0,052	0,143	±12,1%	0,050	0,140	±12,1%
II. Mule antialbumin (0,5 ml, 1 : 2 dil.)									
Donkey alb....	0,025	0,167		0,024	0,169		0,025	0,170	
Mule « ...	0,020	0,175	0,186	0,021	0,179	0,188	0,021	0,179	0,188
Horse « ...	0,045	0,206	±10,2%	0,046	0,207	±10,1%	0,046	0,207	±10,1%
III. Horse antialbumin (0,5 ml, 1 : 4 dil.)									
Donkey alb....	0,020	0,177		0,020	0,177		0,020	0,177	
Mule « ...	0,030	0,157	0,163	0,030	0,157	0,160	0,030	0,157	0,160
Horse « ...	0,045	0,149	± 8,6%	0,034	0,144	±10,6%	0,034	0,144	±10,6%

The data in Table IV reveal that from the antisera tested, homologous and heterologous antigens could carry down almost equal amounts of antibody (±8,6—12,1 per cent). Mule antialbumin contained considerably less antibody than did the donkey or horse antialbumins. The values calculated per 1 ml of undiluted serum were 0,744, 1,320, and 1,304 mg of antibody N, respectively. A complete absorption of all three antisera required far more horse serum albumin than donkey or mule albumin and almost just as much or less donkey albumin than mule albumin. This fact indicates that our horse serum albumin preparation possessed a smaller number of determinant radicals responsible for antigenicity (as calculated for the total N content) than either the donkey or the mule serum albumin preparation.

Discussion

In semi-quantitative and quantitative precipitation tests it could be shown that the donkey, mule and horse serum albumins may be considered homogeneous, immunologically identical antigens. There were, however, certain quantitative differences in reaction between the single systems. The values of the constants of the different equations reflect truly the quantitative differences in the binding capacity of the different albumins and antialbumins. It is known that the value of the constants a and c is determined by the quality of the antigen and the antibody tested, while the value of b and that of d are influenced not only by the quality, but also by the quantity of antibodies. For this reason more reliable results for comparison will be obtained if the values are calculated for a 1,0 mg of antibody-N/ml level (the corresponding antibody-N and antigen-N values are divided by the maximum value of antibody-N of the serum), and the equations are set up on the basis of the values so obtained.

On the basis of the above calculations, the equations for our systems are modified as follows.

I. Donkey antialbumin.

A) + donkey albumin :
1. Antibody-N_{ppt} : $9{,}9\,x - 24{,}5\,x^2$, 2. Antibody-N_{ppt} : $14{,}8\,x - 22{,}0\,x^{3/2}$

B) + mule albumin :
1. Antibody-N_{ppt} : $9{,}2\,x - 21{,}2\,x^2$, 2. Antibody-N_{ppt} : $13{,}8\,x - 19{,}7\,x^{3/2}$

C) + horse albumin :
1. Antibody-N_{ppt} : $5{,}5\,x - 7{,}8\,x^2$, 2. Antibody-N_{ppt} : $8{,}4\,x - 9{,}4\,x^{3/2}$

II. Mule antialbumin.

A) + donkey albumin :
1. Antibody-N_{ppt} : $13{,}9\,x - 47{,}6\,x^2$, 2. Antibody-N_{ppt} : $20{,}7\,x - 36{,}2\,x^{3/2}$

B) + mule albumin :
1. Antibody-N_{ppt} : $17{,}2\,x - 74{,}0\,x^2$, 2. Antibody-N_{ppt} : $25{,}8\,x - 50{,}4\,x^{3/2}$

C) + horse albumin :
1. Antibody-N_{ppt} : $9{,}1\,x - 20{,}3\,x^2$, 2. Antibody-N_{ppt} : $13{,}5\,x - 19{,}1\,x^{3/2}$

III. Horse antialbumin.

A) + donkey albumin :
1. Antibody-N_{ppt} : $17{,}6\,x - 77{,}4\,x^2$, 2. Antibody-N_{ppt} : $26{,}4\,x - 52{,}2\,x^{3/2}$

B) + mule albumin :
1. Antibody-N_{ppt} : $10{,}4\,x - 27{,}0\,x^2$, 2. Antibody-N_{ppt} : $15{,}6\,x - 23{,}7\,x^{3/2}$

C) + horse albumin :
1. Antibody-N_{ppt} : $8{,}5\,x - 18{,}5\,x^2$, 2. Antibody-N_{ppt} : $12{,}9\,x - 17{,}8\,x^{3/2}$

In this way we could eliminate the influence which the amount of antibody in the sera exerts on the constants of the equations. In the above equations the values of the constants are determined exclusively by the quality of antibodies and of antigens. The greater the ability to combine, the higher the value of the constants in the corresponding equations [11]. In other words, higher constants indicate that in the zone of antibody excess more antibody can combine with a given quantity of antigen, or in the zone of antigen excess more antigen can combine with a given quantity of antibody, than in systems with lower constants.

The above equations reveal that the donkey antialbumin tested could combine in an almost equal measure with donkey and mule albumins, but in a considerably lesser degree with horse serum albumin. The explanation of this phenomenon is either that the antibodies produced against donkey serum albumin contain less groups capable of reacting with horse serum albumin, or the quality of the horse serum albumin preparation. The latter seems the more probable, since our horse serum albumin yielded very low constants in tests against the mule or even against the homologous antialbumin, as compared with the values formed in other systems. Further, the equations also indicate that mule antialbumin could unite best with mule albumin, while horse antialbumin was the most effective against donkey albumin.

The described properties of the above systems are characteristic for the albumin preparations and antisera tested. It is known that both quality and avidity of antibodies are highly dependent on the mode of immunization, on the doses employed, on the duration of the immunization cycle and on the individual properties of immunized animals [4, 9, 12—14]. Preparations of antigen, prepared from the same starting material by various methods, or even those produced by the same method, but bearing different production numbers, may vary in binding power [15—17]. Further investigations are required to elucidate whether the homologous and heterologous reactions between donkey, mule and horse serum albumins and the corresponding antisera will show the same peculiarities under variable experimental conditions.

Summary

Crystalline serum albumin from closely related species, donkey, mule and horse, have been examined for immunological relationship in semiquantitative and quantitative precipitation tests. The serum albumins tested proved to be homogeneous, immunologically identical antigens. Minor quantitative differences could be recorded between the reactions given by the systems tested.

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ИММУНОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ АЛЬБУМИНОВ ОСЛИНЫХ, МУЛОВЫХ И ЛОШАДИНЫХ СЫВОРОТОК

Ш. БОЖОКИ и Ф. АНТОНИ

Резюме

Авторы изучали иммунологическую связь между кристаллическими сывороточными альбуминами трех близких видов животных: осла, мула и лошади, частью полуколичественными способами (метод нейтрализации Кюльбертсона, определение оптимальных пропорций Дина и Вебба), частью введенным Гейделбергером и Кендалем методом количественной преципитации, с применением спектрофотометрии Бекмана, модифицированной Житлином.

Исследуемые кристаллические сывороточные альбумины оказались гомогенными антигенами. При истощении любого альбумина любым антиальбумином одновременно полностью удалялись из сыворотки также и реагирующие с другими двумя альбуминами антитела. Следовательно, в отношении иммунной специфичности сывороточные альбумины осла, мула и лошади могут считаться тождественными антигенами.

Реакции исследованных гомологичных и гетерологичных систем — на зоне преобладания антител до зоны эквивалентности — могли характеризоваться общепринятыми уравнениями следующего типа:

1. Антитела — N в преципитате $= 2Rx\frac{R^2}{A}x^2$ или

2. Антитела — N в преципитате $= 3Rx\sqrt{\frac{R^3x^3}{A}}$ где

x = осажденный (добавленный) антиген — N, A = антитела — N, осажденные на какой-нибудь конечной точке зоны эквивалентности (в зависимости от исследуемой системы), R = пропорция антител — N и антигена — N в преципитате, образованном на этой точке.

По данным количественных реакций преципитации, из исследованных антисывороток могли быть получены антитела в почти одинаковом количестве с гомологичными и гетерологичными альбуминами (± 8,6 – 12,1%). Между связывающей способностью различных антисывороток и антигенов наблюдались небольшие количественные разницы.

DISSOCIATION OF B. MEGATERIUM ASSOCIATED WITH THE CHANGE OF THE CELL WALL ANTIGENIC STRUCTURE

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With a view to producing vitamin B_{12} by way of fermentation, 150 different strains of *B. megaterium* isolated from animal excrements and soil samples have been investigated during the last years, and about twenty of them studied permanently for their cultural properties. It is known [1, 2] that slight differences in cultivation of *B. megaterium* are greatly influencing the colonial appearance of this organism. The tendency of *B. megaterium* to form colonies widely varying in appearance is very marked. Serial transfer of this bacterium under seemingly identical conditions does not warrant a uniform appearance of colonies in the subcultures [3]. Though the variants arising in dependence on conditions of cultivation are generally of a very labile character, we have succeeded in observing a dissociation phenomenon in which the new variant differed from the original strain by well-definable, stable, hereditary properties. In the following, a description will be given of the labile variants of *B. megaterium* and the said dissociation phenomenon.

Material and methods

B. megaterium strains. The strains were isolated on meat infusion broth agar from suspensions of faecal and soil samples that had been kept in a water bath at 80° C for 5 minutes. Apart from a few exceptions, the cultures were incubated at 37° C for 18 to 24 hours. Preliminary identification of the freshly isolated strains was primarily based upon their morphological characteristics. In addition to this, culturing them on the synthetic agar recommended by LEWIS et al. [4] also served their preliminary identification. The ability of strains to utilize ammonia as a sole source of nitrogen is considered a significant point in distinguishing them from *B. cereus* [5]. Most of the strains isolated by us were studied in fermentation experiments without going into their detailed identification. Complete identification was limited to strains which form the subject of the present paper, and was based on the criteria described by SMITH et al. [6]. A further means of identification was the demonstration of the presence of *D*-glutamic acid-polypeptide (GAP) in the capsule of the bacteria. An additional facility in differentiating members of the genus *Bacillus* was to examine the strains for their sensitivity to megacine [7, 8] and lysozyme. The method applied was the one described in a previous publication [9]. The lecithinase test [10] was also applied.

Of the standard laboratory strains only the one labelled NRL-B-938 [4] was included in these studies. Some of the twelve strains investigated in detail had been isolated in 1952, some others in 1953. First they were maintained on HM agar, and later on YC-agar. Isolates were taken from time to time from the original spore suspensions and compared with the stock culture. The spore suspensions had been obtained from cultures grown on Tarr's agar and, after washing in saline, stored in the frozen state.

Culture media. Most of our media were made of horse meat extract-pepton broth. Minced horse meat was extracted with two parts of tapwater in a refrigerator, and then boiled. To the extract 1 per cent of pepton («Richter, Budapest») and 0,5 per cent of NaCl were added. The pH was adjusted to 7,4.

a) HM agar (horse-meat agar) was prepared from the above described meat infusion with 2 per cent agar added.

b) HMS-10 agar (horse-meat agar containing 10 per cent sucrose); horse meat extract-pepton broth was converted by adding 4 per cent agar into agar medium and distributed into portions of 100 ml each, and so sterilized. To prepare plates, 100 ml of 20 per cent sucrose were added to the melted medium and poured into Petri dishes. While containing a high amount of carbohydrate, this nutrient medium is comparatively poor in nitrogenous substances and electrolytes. It needs to be mentioned that the sucrose used in the experiments was refined granulated sugar of about 99 per cent purity.

c) HMG-0,4 culture medium was prepared by adding 2 ml of 20 per cent glucose solution to 100 ml of melted HM-medium containing 2 per cent agar. The sugar concentration of the medium was 0,4 per cent.

d) YC medium (yeast extract and casein hydrolyzate) see description in previous publication [8].

Immune sera. Glutamic acid-polypeptide antibody (anti-GAP) was prepared in rabbits immunized with killed suspension of encapsulated *B. anthracis* [11].

To obtain *B. megaterium* immune serum, a young bouillon culture of strains killed with formaldehyde was injected intravenously into rabbits. Inoculating from carefully selected colonies into meat infusion, the culture was aerated by shaking during incubation at 32° to 35° C for 5 to 7 hours. Such cultures had an optical density of 0,4 to 0,6. Details of cultivation and measurement of optical density are to be found in a previous communication [8]. Cuvettes of 1 cm thickness were used to determine optical density in the tests described in the present paper. Adding 0,25 per cent formaldehyde to the young cultures, they were kept in the refrigerator for a day. After centrifuging, the sediment was taken up in 1/10th the volume of saline. Daily injections of 0,5 to 2 ml of the vaccine were administered to the animals on five consecutive days, followed by a week of rest. The rabbits were regularly weighed, and on observing a heavy loss of weight the dose was reduced. Blood samples were taken at the end of the 5-day intervals with a view to controlling immunisation. As soon as the agglutination titre of the blood serum had reached the value of 1 : 800, the animals were bled. The Seitz-filtered sera were stored in the frozen state.

Agglutination test. None of the agglutination tests with *B. megaterium* described in the literature appears to be satisfactory. In fact, MILLER and GOEBEL [12] recently stated that agglutination has failed altogether as a means by which to study serologically lysogenic and indicator strains of *B. megaterium.* Suspensions made from agar slant cultures were found to be unsatisfactory because of their low stability. On the other hand, reliable results were obtained with agglutination tests carried out in the following manner.

The strain to be examined had been plated on HM or HMS-10 agar, and next day the isolated colony was transferred to a meat infusion broth. Of the broth 20 ml were put in a 100 ml Erlenmeyer-flask and incubated, with shaking, at 35° C for 4 to 6 hours. The culture was then diluted with saline until its optical density had become 0,3. Each ml of such suspensions yielded from $2{,}5 \cdot 10^7$ to $3 \cdot 10^7$ colonies on the surface of agar media. A serial twofold dilution was made of sera in tubes measuring 10 mm by 100 mm. The volume of each dilution was 0,5 ml. Thereafter, 0,5 ml of bacterial suspension of standard density were measured into each of the tubes. The tubes were gently shaken when reading the results.

Preparation of lysozyme-digested bacterial suspensions. In essence, SALTON's method [14] was followed in preparing suspensions of bacteria deprived of cell wall. From the fresh suspension of an optical density of 0,6, prepared as above, 15 ml were dripped into an equal amount of vividly boiling saline, taking care that the liquid should not cease boiling during the process. Heating took not more than 30 to 40 seconds. The suspension was then rapidly cooled, crystalline lysozyme (0,1 mg per ml) together with a few drops of toluol, were added to it and kept at 37° C for 14 to 16 hours. Suspensions so treated gave no cell wall staining (Plate VI, fig. 34).

Technique of the capsular reaction. The strain to be examined was suspended in saline. The optical density of the suspension was about 0,5 to 0,6. Placing a droplet of the suspension on a slide, a loopful (0,02 ml) of immune serum was admixed. Examination was made with phase contrast optics, ×90 lens, ×15 compensated eyepiece.

Bacterial morphology. Whenever it was important to preserve natural conditions, cultures grown in YC media not more than a few hours old, were used. The surface of agar-gel plates prepared with saline was covered with the culture, slightly dried and blocks cut from the agar.

With their inoculated surface downwards, the blocks were then carefully placed on slides and immersed in Bouin's fixing fluid. Two hours later, the agar block was carefully lifted off the slide, thoroughly washed and stained. To reveal the cell wall, the preparation was treated with tannic acid and stained with methyl violet, as recommended by GUTSTEIN [13].

Results

Properties of »wild« B. megaterium strains occurring in nature

The strains that after isolation had retained their original properties and which in the following will be referred to as wild (*w*) strains, produced colonies widely varying in appearance, dependent on the nutrient medium. Strains isolated from material found in nature were invariably capsulated. This had already been pointed out [3], and has been confirmed by the present experiments. It is the extent of capsulation, the disintegration and autolysis of the bacteria, that determines the appearance of colonies. With t e increasing effect of any one or more of these factors, the appearance of the colonies varies on different culture media. Even colonies obtained from the vegetative bacilli or spores of the same parent strain may display subtle outward differences. For instance, capsulation was found to be more advanced in one culture than in the other, although both were grown on HM agar prepared in exactly the same way, though on different occasions. Accordingly has varied the ratio of mucoid and dry or partially mucoid colonies (Plate II, fig. 10). Neglecting these subtleties, colonies of wild *B. megaterium* strains obtained in 24-hour cultures at 37° C can be characterized as follows.

On HM agar, mildly mucoid colonies are especially frequent on plates seeded with samples of pasteurized soil or animal faeces. While most of the colonies measure 2 to 3 mm in diameter, in a limited number there also occur midget colonies (diameter, 0,5 to 0,8 mm). Most frequent among the colonies are those with entire margins and a creamy white colour. In some of the strains the margins are somewhat irregular. As a rule, the surface is convex, bright, and has a decidedly mucoid appearance, but there also occur flattened colonies with surfaces of a dull lustre, and occasionally the surface may be quite rough. Sometimes the last-mentioned two properties combine in the same colony; a somewhat dry marginal and a glistening central part of mucoid character.

On YC agar. Colonies from 3 to 6 mm in diameter, with occasional midget colonies. Circular, entire, white, structureless, and uniformly opaque, convex colonies. Surface glistening or dull. Central part sometimes umbonate or umbilicate (Plate I, Figs. 3 and 4).

On HMG-0,4 agar : Colonies measuring 2,5 to 3,5 mm in diameter. Those with intact margins are usually of mucoid character. Surface convex and glistening. Central part in some of them umbilicate (Plate I. Figs. 5 and 6).

On HMS-10 agar. Colonies of wild strains sometimes display extravagant features different even within the same parent strain. Colonial appearance

depends, among others, on some uncontrollable factors of the nutrient medium. At the beginning of this study, convex colonies, of soft consistency, and decidedly mucoid, 3,5 to 4,5 mm in diameter, had been encountered. These traits had been believed to be characteristic of this culture medium. Later, from August, 1953, the colonies, though grown on media prepared in exactly the same way as before, were observed to measure from 6 to 8 mm in diameter ; also, they were succulent and no longer mucoid, but rather viscously semifluid. Sometimes, when the plates were tilted, a thin turbid liquid trickled from the base of the colonies. The most striking feature of these colonies was that, with the main body being more or less translucent, their centre was vitreous and completely transparent. (Plate II, Fig. 11 and Plate III, Figs. 14 and 16.) If allowed to stand at room temperature for a longer period of time, the centre of these colonies displayed a gap penetrating into the nutrient medium (Plate II, Figs. 12 and 13). On examining such cultures under the microscope it was found that their margin consisted of a mass of closely packed viable bacteria, whereas in the vitrous centre there was an amorphous substance with comparatively few unimpaired organisms observable in it. In stained preparation made of the vitreous centre of colonies, bacteria showing various degrees of autolysis and disintegration were seen.

Endeavouring to find out why the appearance of the colonies had changed at a certain period, it was discovered that this was dependent on the agar used for preparing the culture media. Up to the time when it was first noticed that the centre of the colonies is entirely transparent, a yellowish agar-agar had been used, and none of the cultures grown on it had shown a vitreous deterioration in the central part of the colonies. This phenomenon presented itself only when we began to use a fresh stock of recently purchased snowy white agar-agar. Both charges of agar, purchased in the Soviet Union, were preparations made from pelagic algae of the Pacific Ocean. We then prepared HMS-10 culture media both with «yellow» and «white» agars and, observing the absence of the said phenomenon in the former and its presence in the latter medium, we were satisfied that the difference was indeed due to the different quality of the agars (Plate III, Figs. 16 and 17).

The vitreous deterioration of the central part of the colonies, as observed in cultures grown on HMS-10 agar, is somewhat similar to the phenomenon described by TWORT. It cannot be attributed either to temperate phages or bacteriocine. Saline extracts of the cultures showed neither phages nor megacine.

When the suspensions of strains cultured on HMS-10 agar were examined under the phase contrast microscope, highly refractive granules were observed to emerge from the intensively disintegrating bacteria (Plate IV, fig. 21). Their nature is as yet unknown.

It was remarkable that necrosis of the central part of the colonies went hand in hand with an erosion of the nutrient agar. When the cultures had been

washed off with a water spray, there appeared erosions on the surface of the medium at the site of the colonies, especially at their centre (Plate III, Fig. 18). This erosion appeared only on those plates in which the centre of the colonies was vitrously transparent, and no such hydrolysis of the agar could be observed in any other case. It has been assumed that there exists a close correlation between the rapid and intensive necrosis of the central part of the colonies and the erosion of the surface of the nutrient agar medium.

The colonial appearance of wild *B. megaterium* strains is influenced not only by known differences in the composition of culture media but also by subtle, undefinable factors. Batches of media of identical composition frequently gave rise to differences in colonial appearance of *B. megaterium*. Variations of this kind are, however, not hereditary. Subcultures of midget colonies, for instance, contain large colonies and vice versa. If colonies of a characteristically new appearance grown on HMS-10 agar are transferred to another kind of culture medium, the original properties will return. It has been frequently observed that, in the course of serial transfers of a strain, the progeny formed mucoid colonies in some of the instances and dry colonies in others. Such variations are, therefore, of a labile character, and depend on environmental conditions. Beside these merely phenotypic variations of the wild *B. megaterium* strains, it was also possible to observe dissociants, the properties of which, while different from those of the parent strain, proved to be hereditary.

Dissociation of B. megaterium

In the above, the cultural properties of *B. megaterium* as occurring in nature has been briefly characterized. From some of the investigated strains mutants* were successfully isolated that showed properties which were different from those of these wild strains, and were hereditary. From some of our strains colonies were observed to dissociate which, while having much the same appearance as the parent colonies, were formed of cells differing in both morphology and antigenic structure and proved to be hereditary. The recognition of dissociated mutants requires some experience, as the labile appearance of wild colonies and the variety of mutant colonies make it rather difficult to distinguish parents

* Discontinuous variants isolated in the course of dissociation are termed «mutants» in the present paper. By the use of this term it is intended to convey that these variants must not be regarded as the labile state of original («wild») strains produced under the sole influence of environmental conditions. In this case variation is correlated with changes in the hereditary properties of the bacterial culture. It is not yet known to what extent the «mutants» described cover the concept of mutants in the sense of «classical genetics», and therefore the term has to be regarded as an arbitrary one.

from mutant progeny. These differences may be conspicouns on HMS-10 agar, yet as a rule this medium is not suitable for the isolation of mutants, at least not in a direct way, for it is more toxic to mutants than to wild bacteria, therefore sensitive mutant colonies are suppressed if dissociation is not marked. Dissociation is best recognizable on HMG-0,4 agar plates ; subtle intercolonial differences are indicative of dissociation.

The phenomenon of dissociation was first observed on HMS-10 agar in strain No. 111 that had been kept for years on nutrient medium. Not being aware, at the time, of the significance of the phenomenon, no particular care was taken to maintain the original strain, so that the wild bacilli were lost in the course of repeated subcultures and only the mutants survived. More will be reported on them elsewhere.

Dissociation in our strains has been studied for years. While it was repeatedly possible to isolate mutants from some of them, others resisted such attempts. In some strains, such as the above-mentioned strain No. 111, and another one labelled 207, dissociation was considerable and no difficulty was encountered in isolating the mutant. On the other hand, there were strains such as those labelled 208 and 209 of which many hundreds of colonies were examined without discovering a single mutant ; it was only after repeated transfers that one could at last be isolated. Isolation of mutants in these cases was found to be easier if the spore suspension of the wild strains had been inoculated into agar slopes containing 10 per cent Witte's peptone, incubated at 30° C for a couple of days, and so plated. A bacterial layer on the peptone-agar which is inhomogeneous, more translucent than its surroundings, and shows occasional depressions, always points at the presence of mutants. There is usually a considerable number of mutant colonies to be found in subcultures grown from such areas.

Again, there were strains, namely 7 out of the closely studied 12, in which all our attempts to induce dissociation have failed. For instance, no dissociation could be observed in strain No. 216, although the spores had been subjected to ultraviolet irradiation. The spore suspension of this strain contained $5 \cdot 10^7$ potentially germinative spores ; it was washed with saline, and exposed to ultraviolet light (for the technique of irradiation, see our previous publication [8]). Irradiation lasting four minutes reduced the number of spores to $17 \cdot 10^3$ (a killing of 99,964 per cent). From the irradiated substance transfers had been made to HMG-0,4 agar plates and about 2500 colonies were examined, yet no mutants could be detected.

The appearance of mutants can by no means be ascribed to a contamination of the isolated strains, for dissociation has occurred also in colonies that had been obtained from the 10th or even the 20th subculture of the original isolate. So far, the mutants of 5 wild strains have been studied in detail ; they are described in the following.

Cultural properties of mutants, and the technique of their isolation

The 24-hour cultures of the examined 5 strains, namely of the mutants 111 *m*, 202 *m*, 207 *m*, 208 *m*, and 209 *m*, grown on different agar media, showed the following features.

On HM-agar. Colonies 2 to 3 mm in diameter ; margin slightly crenate ; surface more or less convex, according to strain, and slightly or decidedly rough. Protruding centre of mucoid character not infrequent. Some colonies reveal a finely granular structure in transmitted light, but the majority is homogeneous and opaque.

On YC agar. Colonies from 3 to 4 mm mingle with occasional midget colonies (0,5 mm). All are uniformly opaque and have entire margins. Among mucoid or smooth surfaced colonies there occur some with umbilicate or umbonate centres (Plate I, Fig. 8).

On HMG-0,4 agar. Colonies 2 to 3 mm in diameter, and slightly structural in transmitted light. Margins somewhat uneven. Surface dully lustrous or moderately rough. Colonies with bright, mucoid, umbilical centres also occur.

On HMS-10 agar. Diameter from 1,5 to 2,5 mm ; structural in transmitted light ; margin crenate ; surface rugose (Plate III, Fig. 15).

If cultured on HM agar, no essential difference between wild and mutant bacteria can be perceived. The differences are more conspicuous on HMG-0,4 agar, especially as regards the marginal parts of the colonies and their appearance in transmitted light. Differences are best revealed in cultures grown on HMS-10 nutrient medium, which, while valuable for separation of mutants, cannot, for the reasons given above, be used for their direct isolation. The most expedient procedure for isolating mutants was found to be the following.

Five to six HMG-0,4 agar plates are seeded with spore suspensions prepared on TARR's agar. Plating may be preceded by culturing on peptone agar, as already mentioned. In accordance with the above, colonies of the «rough» type will have to be looked for. It should, however, be borne in mind the rough character does not assert itself in all mutant colonies, therefore much attention has to be devoted to the margins and the occasionally granular structure of the colonies. All colonies suspect of being mutants should be transferred onto HMS-10 agar on which it is easy to distinguish between wild and mutant types.

It must be mentioned that with strains showing moderate dissociation some of our experiments have failed in isolating the mutant.

Antigenic structure of mutants

Table 1 shows the results of agglutination tests made with blood sera of rabbits immunized with wild and mutant strains.

Table I

Agglutination by different immune sera

Serum	Results of agglutination tests with different suspensions								
	202 *w*	202 *m*	207 *w*	207 *m*	208 *w*	208 *m*	209 *w*	209 *m*	111 *m*
202 *w*	800	—	800	—	—	—	—	—	—
202 *m*	—	800	—	—	—	800	—	800	800
207 *w*	400	—	800	—	—	—	—	—	—
207 *m*	—	—	—	800	—	—	—	—	—
208 *m*	—	400	—	—	—	800	—	400	800
209 *w*	—	—	—	—	—	—	800	—	—

Legend: Figures mean the reciprocal value of the agglutination titre. In negative tests no agglutination was obtained even in 1 : 50 dilutions of serum.

It was unfortunately not possible to extend the studies to the homologous sera of strains 208 *w* and 209 *m*, as all animals in which it had been attempted to produce sera with these strains fell victim to the energetic immunization.

The agglutination tests undertaken appear to justify the following statements. Sharp serological differences exist between the individual wild strains. Thus, for instance, there is no cross reaction between strains 202 *w*, 209 *w*, and 208 *w*. On the other hand, strains 202 *w* and 207 *w* are similar or even identical in antigenic structure. It is not intended to deal in this paper with the antigenic structure of wild strains; experience obtained on a wider range of material will be reported elsewhere. A striking feature is that the serologically different strains 202 *w*, 208 *w* and 209 *w* gave rise to mutants of identical antigenic structure, while the mutant strains 207 and 202 of different antigenic structures were derived from serologically similar wild strains. It can be seen that homologous sera of strains 202 *m* and 208 *m* agglutinate strain 209 *m*. On the other hand, the high specificity of strain 209 *w* is remarkable. Accordingly, the antigenic structure of the mutant seems to be independent of that of the parent strain.

The result of the agglutination test was not affected by whether a suspension heated to 80° for 15 minutes or to 100° for 2 minutes had been used. From this it follows that a thermostable antigen is responsible for the specificity.

Agglutinated wild and mutant strains differ in appearance. The difference is particularly striking if after transfer of the suspension the tubes are shaken for 5 to 10 minutes and thereafter allowed to stand in the refrigerator for two hours. Upon the effect of their sera, the bacteria of mutant strains clump into large loose flocks, while agglutinated wild strains have a rather granular character.

The cells of the bacterial suspensions used in the agglutination tests were not capsulated. It was demonstrated by SALTON [14] that the cell wall of

heat-killed *B. megaterium* can be completely detached with the aid of lysozyme. On treating first with heat and then with lysozyme, turbidity in the bacterial suspensions decreased by one half without, however, a dissolution of their cytoplasm. Reduction of density is thus correlated with the digestion of the cell wall. Performing this experiment with filamentous mutant cultures, the filaments are seen to disintegrate into single bacilli which are, however, no longer positive to GUTSTEIN's cell-wall stain, but otherwise retain their shape unchanged (Plate VI, Fig. 34).

Table 2 illustrates the results of experiments made with suspensions subjected to rapid heating and then treated with lysozyme.

Table II

Agglutination tests with heat-killed and lysozyme-treated suspensions of strains 208 *m and* 111 *m in the presence of* 208 *m serum*

Strain	Treatment	Dilution of serum (reciprocal value)					
		50	100	200	400	800	Control
208 *m*	Heat + lysozyme ...	—	—	—	—	—	—
208 *m*	Heat	+++	+++	+++	+++	—	—
111 *m*	Heat + lysozyme ...	—	—	—	—	—	—
111 *m*	Heat	+++	+++	+++	++	—	—

Similar experiments with other wild and mutant strains have unequivocally demonstrated that heat-killed suspensions of *B. megaterium* treated with lysozyme lose their agglutinability.

Immune specificity of the capsule of mutants

It has been mentioned that the agglutination test shows sharp serological differences between the suspensions of non-capsulated wild and mutant strains. The capsule of *B. megaterium* gives the so-called capsular swelling reaction upon the effect of its homologous antibody [15] and upon that of anti-glutamic acid-polypeptide (anti-GAP) [15, 16]. The wild and their corresponding mutant strains give with their homologous sera very specific capsular reactions; this is demonstrated by our experiments made with strains 207 *w* and 207 *m* (Plate V, Figs. 24 to 29). A suspension of the strains cultured on YC agar gave the capsular reaction with a specificity corresponding to that of the agglutination test. It can be seen in Fig. 25 that a capsule with a finely striated structure was made visible in the bacilli by the homologous antibody of strain 207 *w*, while no such capsular reaction was elicited by the anti-mutant serum (Fig.

26). Anti-GAP serum has made a large homogeneous capsule discernible around the bacteria of wild strains. In agreement with the above, serum 207 *m* gives no reaction with bacteria of the corresponding wild strain (Fig. 28), while a capsular reaction is obtained with homologous i. e. the 207 *m* bacteria (Fig. 29). In this strain, a septated capsule around the bacteria becomes distinguishable upon the effect of the serum. The main septa appear at the junctions of the cells. Secondary, thin septa are seen elsewhere in the capsule. Various capsular structures become discernible on mutant bacilli upon the action of anti-GAP serum (Plate VI, Figs. 30 and 31). Along with completely homogeneous ones, a considerable number of cells have structural capsules. The figures illustrate well how the polypeptide capsular substances made visible by the anti-GAP serum are divided into independent parts. On some cells the thin isolated bundles of polypeptide are well descernible while, at the same time, no polypeptide micelles can be seen at the junction of individual cells. Such points correspond to the site of the thick septum that becomes visible upon the action of homologous serum. It is seen from our electron photomicrograph (Plate IV, Fig. 23) that there is no cytoplasm at the junctions of mutant cells, and that only the remnants of the empty cell wall are visible there. From this it follows that the polypeptide produced in the cells does not reach the surface at such points, and explains why only specific substances detached from the remnants of the empty cell wall can be discovered in this area. This is why thick septa appear after treatment with homologous serum, while for the delicate septa the debris of cell walls wedged in between the polypeptide bundles are apparently responsible.

The polypeptide bundles which form the capsular substance of *B. megaterium* do not, of course, reach the surface in a single plane, but in a multiple of planes that corresponds to the entire circumference of the bacteria, and it is for this reason that separate rings formed by the bundles of polypeptide molecules surround the cells. This structural character disappears with the ageing of the cells and the concomitant gradual disintegration of the marginal parts of the polypeptide micelles. In this phase, even on the effect of anti-GAP serum, the picture produced will be that of a capsule homogeneous in appearance (Plate VI, Fig. 30).

It has been established that development and involution was quicker in wild colonies than in their mutants, and that this difference was particularly marked on YC and HMS-10 nutrient agar. This makes it clear why, in the presence of anti-GAP serum and with cultures of the same age, the capsules of wild strains are of a more homogeneous structure than those of the mutant. Obviously, then, only cells of quite young (merely a few hours old) cultures are suitable for demonstration of the polypeptide bundles of wild strains. Using quite young cultures of wild strains in our previous investigations [16], it was possible to demonstrate under the electron microscope structural conditions corresponding to those in the polypeptide frame of which the capsule consisted.

Stability of mutants

As regards reversion of mutants, we could not yet form a definite opinion. In the course of the last two years several hundred samples of our stored spore suspensions of mutants, as also of their cultures, maintained on YC agar have been plated on various, chiefly on YC and HMS-10, agars. With the exception of three cases, a true mutant progeny was always obtained. In the exceptional cases there grew, side by side with hundreds of pure mutant colonies, one or two colonies that were characteristically wild in appearance. This phenomenon was observed on one occasion in each of the strains 202 *m*, 111 *m*, and 207 *m*. Further investigations proved the wild-looking colonies to be wild indeed, inasmuch as their cultures in bouillon media contained vigorously motile rods consisting of one or two members and could be identified as *B. megaterium*. The wild appearance of the colonies obtained from strains 202 *m* and 207 *m* was quite in harmony with the antigenic structure of the cells. The question arose whether these rare cases of «back mutation» could not possibly have been produced by contamination. Having worked in the laboratory with both mutant and wild strains, this possibility cannot be excluded; on the other hand, contamination does not seem probable if we remember that the antigenic structure of the colony isolated from the mutant was the same as that of the original wild strain. Apparently, rare occurrences of back mutation cannot be left unconsidered, and further investigations will have to throw light on this problem.

Discussion

The colonies of *B. megaterium* are described in the literature as widely varying in appearance. Quoting BERGEY's 6th edition on their agar cultures, «Large, smooth, soft convex, entirely opaque, creamy-white to yellow. The rough stage is usually concentrically ridged with a thin edge». Neither this description nor the monograph of SMITH et al. [6] properly explains the meaning of the term «rough stage». It is not clear whether mutants with stabilized properties or labile forms of bacillary colonies dependent on environmental conditions are meant.

A similar ambiguity seems to be patent in respect of the different variants described by KNAYSI [1] in 1933. As far as it can be established from his description, these were not stable either. Incidentally, to none of his variants did he apply the term «rough». WAHL [2] made a study of the lysogen- and phage-sensitive strains derived from the experiments of DEN DOOREN DE JONG [17]; classifying the individual colonies by their appearance, he described them as «S» and «R» variants, but did not regard them as stable, much rather in a

state of continuous transformation into one another, the process largely depending on the composition of the nutritive medium.

The point of departure in this study was the description of the cultural properties of «wild» *B. megaterium* strains as found in nature. It had been pointed out in a previous communication that under adequate conditions of cultivation such strains are invariably capsulated. Extracts from the capsular substance point to the presence of D-glumatic acid-polypeptide, the same as is encountered in the cultures of *B. anthracis* and other species of the genus *Bacillus* [3, 18, 19]. The *B. megaterium* strains studied in the course of the present experiments were no exceptions to this rule, and it has been possible to demonstrate the presence of glutamic acid-polypeptide in the extracts, respectively the bacillary capsules.

In identifying *B. megaterium* strains freshly isolated from substances as found in nature, in addition to the dimensions of cells and spores, it has been accepted as a morphological criterion whether or not the bacilli occurred singly, in pairs, or in quite short chains, and whether on staining after careful fixation the rods had rounded off ends (Plate IV, Fig. 22). Dimensions and the sharp morphological features are important in characterizing *B. megaterium ;* beside the general biochemical properties, it is their markedly specific sensitivity to lysozyme, and especially megacine, which serves as a valuable guide to their identification [9].

Differences in the appearance of colonies grown on different nutritive agar media are less characteristic. The appearance of colonies greatly varies according to the composition of the cultur medium and a number of other factors which we are as yet unable to control. Among the known factors deciding the appearance of a colony, a prominent place is taken by the extent of capsulation, and the involution or complete disintegration of the bacteria in certain, especially the central, parts of the colonies. These, however, can be greatly influenced by varying the composition of the culture medium, e. g. by addition of carbohydrates or of plant extracts poor in carbohydrates (such as tomato serum [16] or mashed potato extracted with 66 per cent ethanol [20]). Other factors, for instance those responsible for midget colonies, are for the present beyond our control.

A rather peculiar form of wild *B. megaterium* colonies was observed in cultures grown on horse meat agar medium containing 10 per cent sucrose, a medium relatively poor in nitrogen and electrolytes. This type of colonial appearance seems to be contingent on moments that still go undetected, *e. g.* the quality of the agar employed. On the nutrient medium in question the very succulent, often semifluid colonies showed a vitreously transparent central part, in which remnants of dead and disintegrated bacteria were found. The rapid necrosis in the centre of these colonies cannot be ascribed to the action of known extracellular agents such as phages or bacteriocine. The rapid necrosis is accompanied by an erosion of the agar surface, and this means that an enzyme causing

the agar to decompose is set free or produced in the course of bacterial disintegration. We are not aware of any data in the literature concerning agar erosion in connection with *B. megaterium*. Under the phase constrast microscope the cells in the necrotized central parts were seen to contain a great number of highly refractive granules. At a later stage, these escape from the cells.

Apart from labile, non-hereditary colonial properties susceptible of being influenced by the composition of the culture medium, dissociants with hereditary properties were also observed, although technical reasons have made it rather difficult to recognize them and may have caused some of our experiments to be failures. Under adequate conditions of growth, five of our wild strains as also their mutant progeny were found to be capsulated and therefore capable of forming mucoid colonies. The capsular frame of these mutants consists of glutamic acid-polypeptide ; in this respect, then, they do not differ from the wild parent strains. Dissociants of this kind often form smooth, bright or even mucoid colonies, and cannot therefore be regarded as R variants in the usual sense of the term. BISSET [21], however, maintains that R variants can no longer be defined on the strength of their colonial appearance alone, as cytologic and division properties are also important characteristics. Without wishing to take sides in the controversy about BISSET's theory on the mechanism of cell wall formation challenged by DAWSON and STERN [22], we share BISSET's views regarding the cytology of rough bacteria. According to those views is the correlation between cell-wall formation and cytoplasmic fission which characterises bacteria of the «rough» type. GUTSTEIN's cell-wall stain and the electron-microscopic examination of one of our strains have made it possible to establish the characteristics which permit our mutants to be regarded as cells of the rough type.

The loss of flagella is another cytologic alteration associated with mutation. Accordingly, the formation of a cell structure of the R type is connected with the usual H—O variation.

A radical change in the antigenic structure of the cell wall is the most characteristic feature of dissociation, as confirmed by agglutination tests made with young broth cultures. Since *B. megaterium* is not capsulated in cultures of this kind, the serological specificity of the bacterial surface is governed by the antigenic structure of the cell wall. Besides, the sensitivity of the thermostable specific substance to lysozyme also points to a connection between agglutination and cell wall substance. Both wild and mutant bacteria cease to be agglutinable after the lysozyme-sensitive cell wall has been depolymerized.

It is an interesting point that, notwithstanding the diversity of the antigenic structure of the different wild parent strains, the antigenic structures of their mutants are often identical, or only slightly different. There are sharp serological differences between individual mutant strains, and it even may happen that immunobiologically identical wild strains have different mutants.

On the basis of our investigations we could not establish a general rule governing changes in serological specificity.

While in the course of dissociation there occurs a profound change in the antigenic structure of the cell wall, the cells of *B. megaterium* retain their ability to continue the production of glutamic acid-polymer, *i. e.* the capsular substance. This seems to be confirmed by the capsular reactions obtained in capsulated mutant and capsulated wild strains. It has been demonstrated by TOMCSIK [15], further by TOMCSIK and GUEX—HOLZER [19], that the capsules both of *B. megaterium* strains and of another not closely identified strain termed «M-bacillus», become visible under the action of anti glutamic acid-polypeptide serum. Capsules thus made visible are not always homogeneous, some of them are structured [16, 23]. The striated structure made visible by anti-GAP serum and the observations made under the electron microscope have allowed the assumption that the frame of the capsules was formed by distinct bundles of the filamentary glutamic acid-polypeptide molecules. TOMCSIK and GUEX—HOLZER described the capsule of the «M-bacillus» likewise as non-homogeneous (23, Plate I, Fig. 5). The picture of this capsule is very similar to that elicited by anti-GAP serum in our mutant strains (Plate VI, Figs. 30 and 31). The structure appears to confirm our assumption of distinct polypeptide micelle bundles.

This and other observations illustrated in the Plates make it obvious that there exists a close genetic relation between the cell wall and the nonpolypeptide parts of the capsules. Assumedly, these parts reach the capsule from cell wall itself, probably from its surface. Evidently, then, the capsule of *B. megaterium* is made up from the polymer of glutamic acid ; this owes its serological heterogeneity to the specific substance which in the course of the capsule's development becomes detached from the cell wall and is, as far as is congruent with the structure and condition of the cell, wedged in between or intermingled with the polypeptide micelles. We attach no primary morphological or functional significance to the capsular structure closely dependent on the condition of the cell ; it is in our opinion just an interesting instance of the dynamic development [24] and state of the bacterial capsule which is governed by biochemical events in the cytoplasm.

The dissociation demonstrated in our experiments seems to occupy an exceptional place among known bacterial mutations. It involves the loss of flagellum and a change in the antigenic structure of the cell wall without, however, affecting the continued production of glutamic acid-polypeptide, the substance of the capsule. Under adequate conditions of cultivation, this could be observed in both wild and mutant strains. In capsular microorganisms, such as the pneumococcus, S→R variation causes the loss of the capsule-forming capacity. On the strength of the cytological characteristics the dissociation observed in our experiments can be regarded as being a case of S→R variation which, in *B. megaterium*, does not involve the loss of capsulation, that is of the production

of glutanic acid polymer but produces a change in the antigenic structure of the cell wall.

While, so far, approximately 150 strains, termed «wild» in this paper, have been isolated from natural sources, mutants of the kind described above could be obtained from cultured strains only. We surmise, however, their occurrence in nature and, knowing their properties, it will certainly be possible to isolate them.

Dissociation as described in the present paper must in no way be associated with lysogenesis. IONESCU has [25] recently reported on changes in the colonies of a *B. megaterium* strain after the bacilli had been made lysogenic by the addition of temperate phage. As soon as the colonies had lost their lysogenic property, their appearance reverted to the original state. The strains studied by us were not lysogenic [8], and to temperate phage was involved in the dissociation.

Summary

1. Depending on the composition of the nutrient medium colonies widely varying in appearance develop from «wild» *B. megaterium* strains occurring in nature. These variable colonial appearances do not however constitute hereditary characters : they are merely phenotypic. As yet uncontrollable factors, as for intance the quality of the agar, exert influences upon them.

2. The outward appearance which varies with differences in the composition of the nutrient medium is governed by the extent of capsulation and disintegration of the cells building up the colonies. On horse-meat agar containing much sucrose, necrosis of the central part of the colonies sets in and owing to disintegration this part becomes vitreously translucent. This phenomenon is accompanied by the appearance of agar-depolymerizing enzymes.

3. A part of the *B. megaterium* wild strains dissociates in subcultures. The arising mutant consists of rods without flagella and placed threadlike. It may be regarded as the R form of *B. megaterium*. Its most characteristic feature is that the antigenic structure of its cell wall is entirely different from that of the cell wall in the original strain.

4. The capsular framework of *B. megaterium* consists of micelles of D-glutamic acid-polypeptide appearing on the cellular surface at intervals. The capsular structure visible upon the effect of immune sera is due to the specific materials detached from the cell wall and filling the interstices among the polypeptide bundles. Serologically this structure differs within the same strain depending on its S or R state. In the present author's opinion no primary morphologic or functional significance attaches to the variable structure of the capsule in *B.*

megaterium, this being rather the consequence only of biochemical processes taking place in the cytoplasma.

The observations under the electron microscope were made by *F. Guba* in the Electron Microscope Laboratory of the Hungarian Academy of Sciences, and we are indebted for his kind assistance.

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Legenda to the Figures

The magnification of photographs of bacterial colonies is × 1.6. Magnification of photomicrographs is indicated in each case.

PLATE I

Figs. 1, 3, 5: Colonies of strain 202 *w* on different culture media. Fig. 1, on HM agar; Fig. 3, on YC agar; Fig. 5, on HMG—0,4 agar

Figs. 2, 4, 6: Colonies of strain 208 *w*. They are rough on HM agar, and mucoid on HMG—0,4 agar

Fig. 7: Strain 202 *m* on HM agar. Colonies are of the rough character. Compare with the colonies of strain 202 *w!* (Plate I, Fig. 1)

Fig. 8: Strain 202 *m* on YC agar. Colonies are intensely mucoid

PLATE II

Fig. 9: Strain 208 *m* on HMG—0,4 agar. Colonies are, in contrast to parents (Plate I, Fig. 1), of the rough type

Fig. 10: Strain 207 *w* plated on HM agar. Most of the colonies belong to the rough type. There are also a few mucoid colonies. (No dissociation; differences in phenotypical appearance)

Fig. 11: 18-hour culture of strain NRL—B—938 on HMS—10 agar. Patches resembling phage plaques are due to necrosis of the central part of colonies (x 0,5)

Fig. 12: Strain 121 *w* on HMS—10 agar. Incubated at 37° C for a day, then kept at room temperature for 6 days. Empty centre of colonies well visible

Fig. 13: Strain 209 *w* on HMS—10 agar. A 7-day culture. Central necrosis of colonies

PLATE III

Fig. 14: Strain 121 *w* on HMS—10 agar. 24-hour culture in transmitted light. Centre of colonies vitreously translucent

Fig. 15: Mixed spore-suspension of strains 207 *w* and 207 *m* on HMS—10 agar. The almost semifluid wild colonies readily distinguishable from the rough colonies of the mutant

Figs. 16 and 17: Colonies of strain 209 *w* on HMS—10 nutrient medium prepared withf different charges of agar. Fig 16, on «white« agar; Fig. 17, on «yellow» agar

Fig. 18: Erosion on HMS—10 agar. 18-hour culture of strain 121 *w* washed with water spray

PLATE IV

Fig. 19: YC broth culture of strain 207 *m*. Gutstein's stain (x 1100)

Fig 20: YC broth culture of strain 207 *m*. Gutstein's stain (x 1800)

Fig. 21: Sample taken from centrally necrotized colonies of strain 207 *w* on HMS—10 agar as seen under the phase contrast microscope (approx. x 2000). Highly refractive granules in and outside of the disintegrating cells

Fig. 22: YC broth culture of strain 207 *w*. Gutstein's stain (x 1800)

Fig. 23: Electron microphotograph of a 6-hour culture of strain 207 *m*. Shadowed with gold (x 5500)

PLATE V

Suspension of 18-hour YC agar culture of strains 207 *w* and 207 *m* in the presence of anti-glutamic acid-polypeptide (anti-GAP), anti—207 *w* serum, and anti—207 *m* serum, as seen under the phase contrast microscope

Fig. 24: Strain 207 *w*, anti-GAP serum (approx. x 2000)

Fig. 25: Strain 207 *w*, anti-207 *w* serum (appr. x 1500)

Fig. 26: Strain 207 *w*, anti-207 *m* serum (appr. x 2000)

Fig. 27: Strain 207 *m*, anti-GAP serum (appr. x 1500)

Fig. 28: Strain 207 *m*, anti-207 *w* serum (appr. x 2000)

Fig. 29: Strain 207 *m*, anti-207 *m* serum (appr. x 1800)

PLATE VI

Figs. 30 and 31: Strain 207 *m*. 18-hour culture on YC agar containing 2 per cent saccharose, in the presence of anti-GAP serum (appr. x 2000). Capsules with different structure visible in the same field

Figs. 32 and 33: Strain 207 *w*. 17-hour culture on HMS—10 agar in the presence of homologous serum. Granular disintegration of cells accompained by dissolution of capsule (appr. x 2000)

Fig. 34: Broth culture of strain 207 *m* after treatment with heat and lysozyme; Gutstein's stain

PLATE I

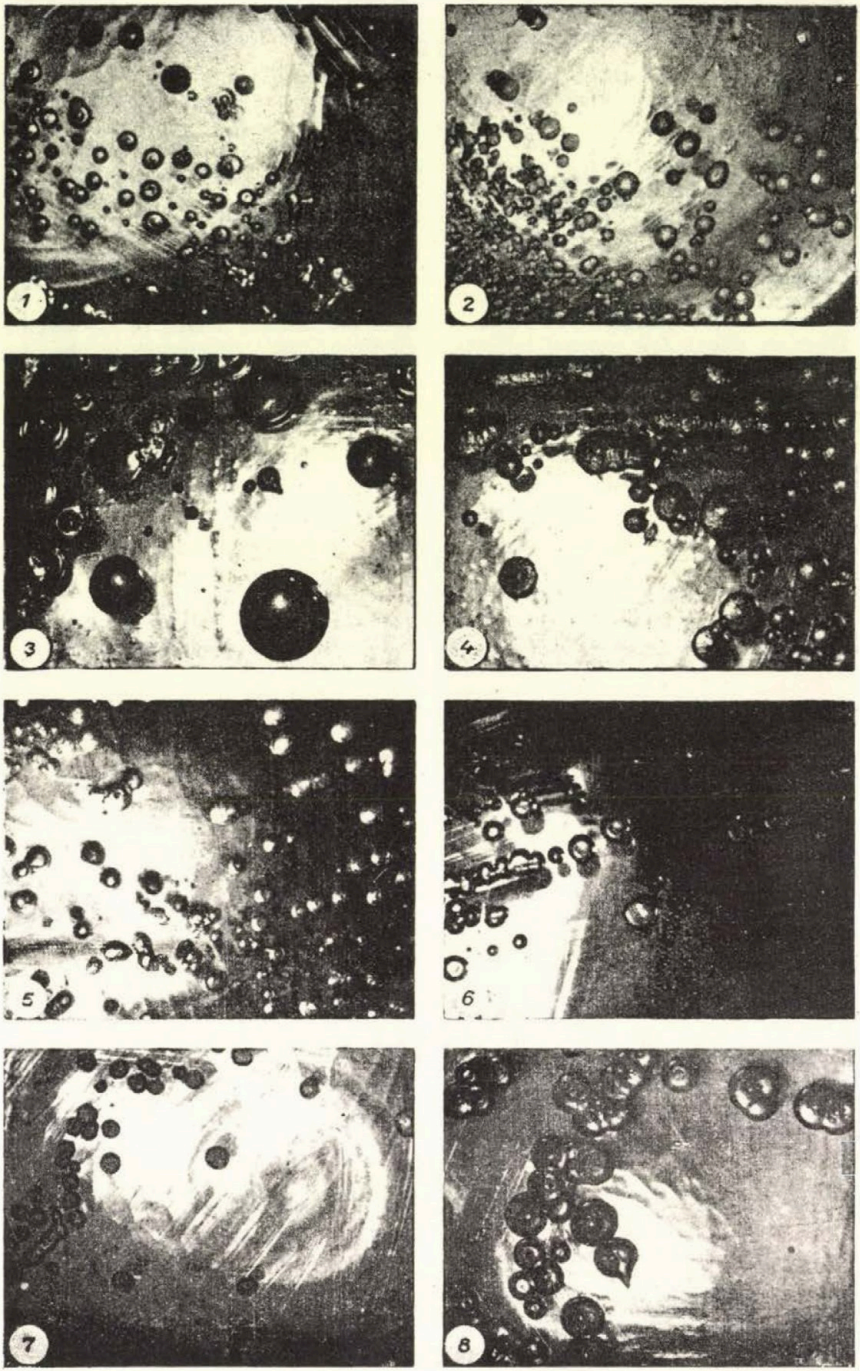
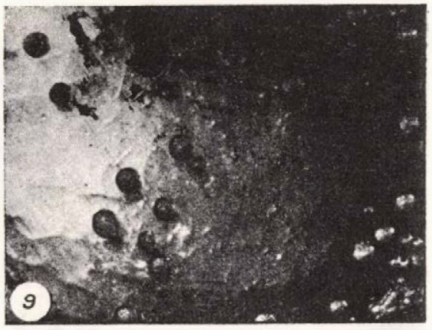
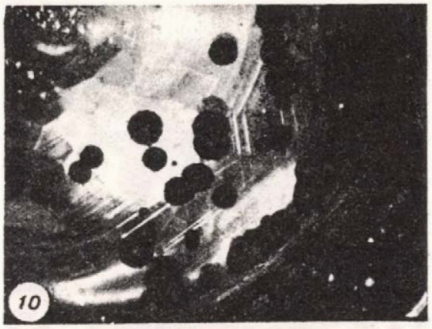


PLATE II





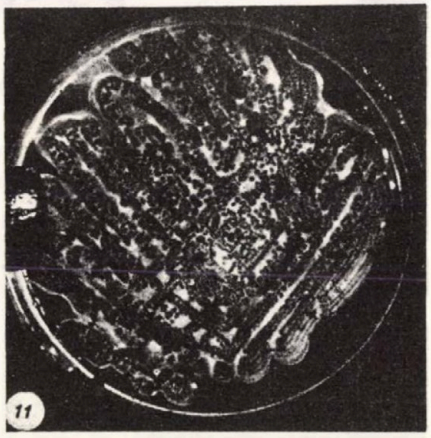


PLATE III

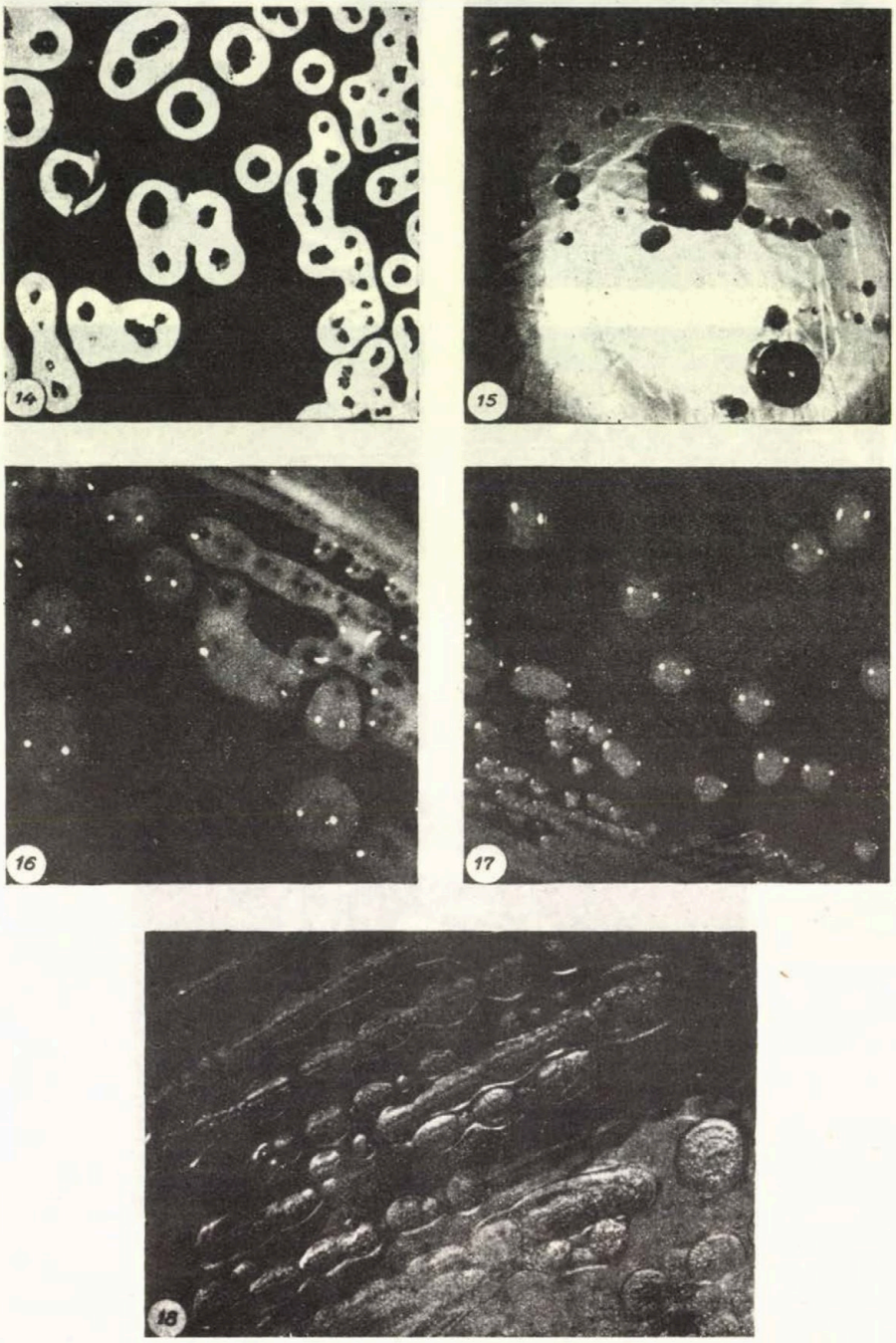
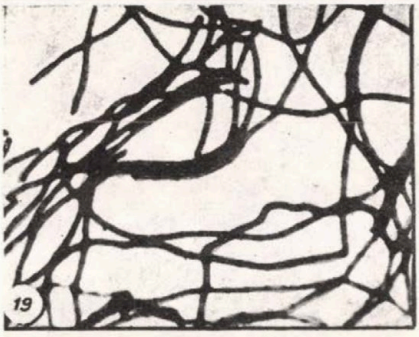
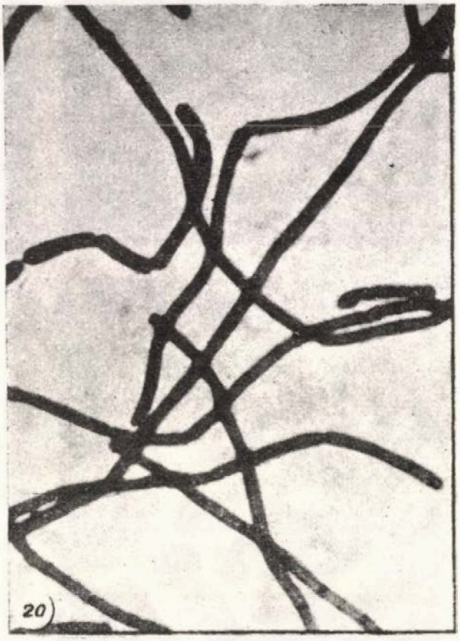
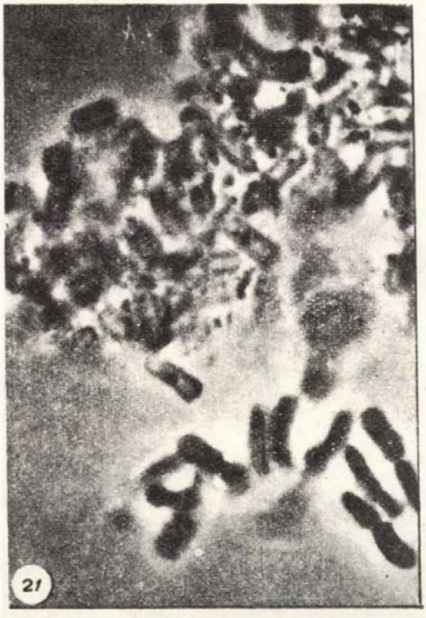
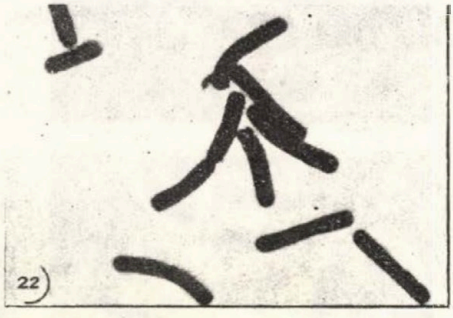


PLATE IV









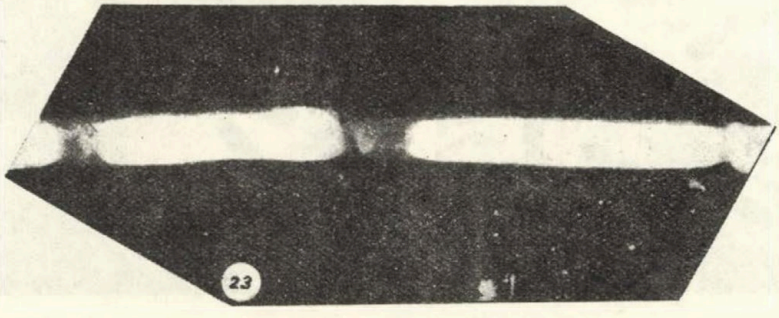
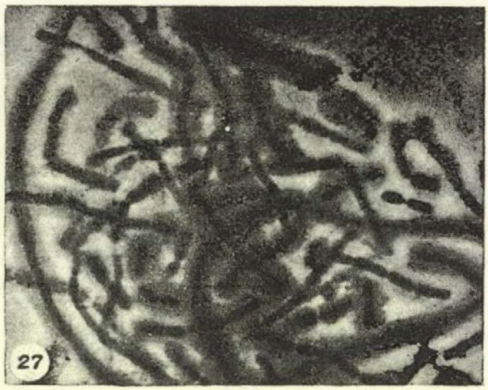


PLATE V



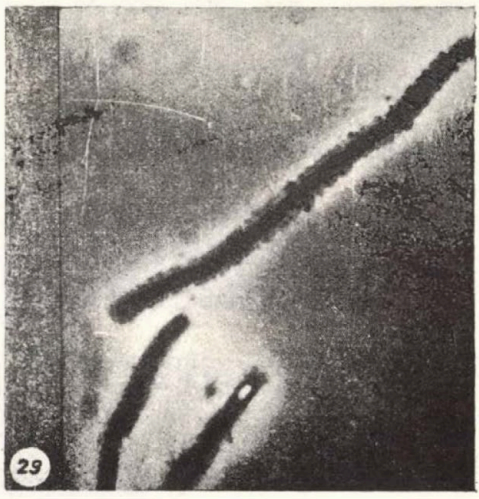
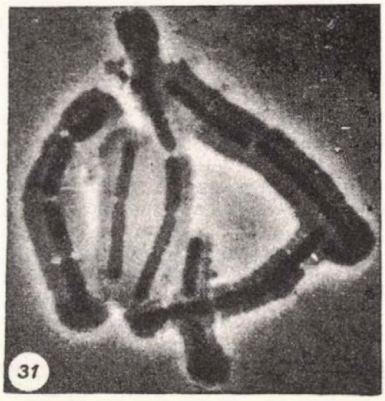
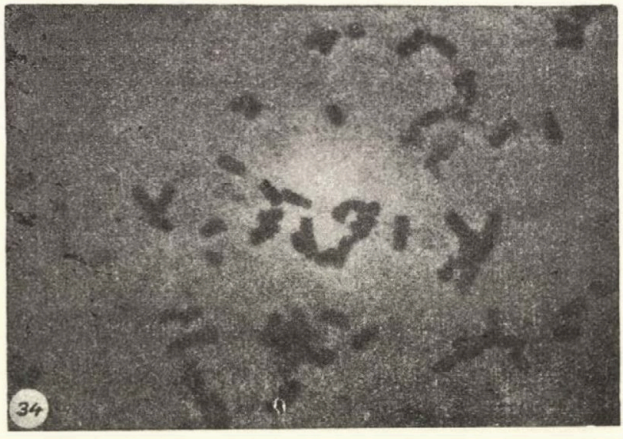


PLATE VI





ДИССОЦИАЦИЯ B. MEGATERIUM В СВЯЗИ С ИЗМЕНЕНИЕМ АНТИГЕННОЙ СТРУКТУРЫ СТЕНОК КЛЕТКИ

Г. ИВАНОВИЧ

Р е з ю м е

1. Колонии встречаемых в природе штаммов B. **megaterium «Wild»** при культивировании на агаре появляются в весьма различной форме, соответственно составу питательной среды. Этот изменчивый внешний вид однако не является наследственным, а только фенотипичным. На внешний вид колоний влияют и не проверимые до сих пор факторы, как, например, качество агара.

2. Зависящий от состава питательной среды различный внешний вид колоний определяется размером образования капсул и дезинтеграции составляющих колонию клеток. В центре колоний, растущих на агаре с лошадиным мясом с большим содержанием сукрозы, клетки отмирают и вследствие дезинтеграции центр колонии становится стекловидным. Этот феномен сопровождается появлением деполимеризующего энзима.

3. При последующих пересевах часть штаммов B. **megaterium Wild** диссоциирует. Образующийся мутант не имеет жгутиков и состоит из нитевидно расположенных палочек. Мутант может быть принятым за *R*– форму B. **megaterium** и наилучше характеризуется тем, что антигенная структура стенок его клеток совершенно различна от таковой исходного штамма.

4. Остов, т. е. основание капсулы B. **megaterium** состоит из мицелиев полипептида D-глютаминовой кислоты, расположенных прерывисто на поверхности клетки. Выявление структуры капсулы под действием иммунных сывороток вызывается специфическими веществами, слущенными со стенки клетки и наполняющими промежутки между полипептидными пучками. Вещества эти являются серологически различными, соответственно *S* или *R* форме одного и того же штамма. Не можем придавать первостепенного морфологического или функционального значения структуре капсулы, зависящей от состояния клеток B. **megaterium** и рассматриваем это только как интересный пример, связанный с динамическим состоянием капсулы бактерии.

VARIATION OF THE CYTOPATHOGENIC ACTIVITY OF AUJESZKY-DISEASE (PSEUDORABIES) VIRUS

By

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(Received, May 16, 1955)

Studies concerned with the propagation of pseudorabies virus (in the following: Ay virus) in tissue elements of chick embryo have been in progress at our Institute for years [1, 2, 3]. The amount of virus was usually estimated by determining the limiting dilution which would initiate cytopathogenic changes in chick embryo tissue cultures. As it has been reported recently [4], the Ay virus showed a peculiar cyclic type of multiplication in cultures of excised chorio-allantoic membrane of chick embryo. The cyclic changes in infectivity of such cultures were observed only in tests for estimating cytopathogenic activity, while they could not be demonstrated by intracerebral inoculation of mice. We have not yet been able to give a satisfactory interpretation of this phenomenon. It has been suggested that during multiplication in excised chorio-allantoic membrane the virus would vary in the sense that the virulence for tissue culture of newly formed viral particles would change within the developmental cycles. Our present experiments have furnished some data indicating that the virulence of Ay virus for tissue culture is highly influenced by the host cells in which the virus propagates. It has been observed, namely, that the intense destructive action of Ay virus on chick embryo cells is lowered by serial transfers in mouse embryo tissue suspension.

Materials and methods

Viral material. The previously employed strain No. XXV. has been used also in these investigations. Brain tissue from infected mice, stored in the frozen state, served as stock virus. The viral material used in the experiments was obtained in tissue suspension of chick embryo. Transfers were made at 2 or 3 day intervals over more than 300 passages. The tissue suspension infected with virus was homogenized and the extract obtained by gentle centrifugation served as viral material. (The viral material thus prepared will be referred to as CEC virus, chicken embryo culture virus, throughout this paper.)

Mouse embryo virus (which will be referred to as MEC virus in this paper) was obtained by a similar technique except that the virus was grown in suspension of finely minced tissue of mouse embryo.

In some of our experiments we have used virus never before cultivated in tissue suspension.

Cultivation of virus in tissue suspension of chicken or mouse embryo. Ten to 13 days old chick and 15 to 19 days old mouse embryos were washed in saline and then, after removing head and limbs, the torsos were finely minced with a sharp pair of scissors. Approximately 200 mg of finely minced tissue was placed in a 100 ml Erlenmeyer flask and 3,6 ml of Simms X_6

solution were added to it. Unlike in earlier experiments, bovine serum ultrafiltrate was not employed since this proved to have no effect whatsoever on the growth of virus. The tissue suspension was then inoculated with 0,4 ml of viral material. The nutrient medium contained 15 units of penicillin and 15 units of streptomycin per ml. The flasks were closed by means of well-fitting cellulose stoppers and incubated at 37° C for from 48 to 72 hours.

Titration of cytopathogenic effect. The cytopathogenic effect of the viral material was determined on explants made either of fragments of embryonic chicken heart or that of mixed tissue of mouse embryo. In our previous experiments minced tissue had been maintained in nutrient fluid containing various dilutions of virus for 3 days and then explanted into hanging drops of chicken plasma. The explants were then enclosed into hollow ground slides. As already mentioned [1], this procedure was not always adequate if embryos obtained during winter were used. The method applied in the present work has eliminated this untoward circumstance and by its use the duration of experiments could be reduced from 6 days to 3.

One drop of hen plasma containing heparin 1 in 50 000 was spread out to form a strip within each 180 mm by 18 mm test tube. Four to six pieces of tissue were placed into each strip, over which 1 drop of embryonic extract was allowed to run down. The tube was then left to lie horizontal for about 15 minutes. When the tissue had been firmly attached to the plasma, 1,8 ml of nutrient fluid were added to the tube. In tubes so prepared, serial dilutions were made of the viral material, directly in the nutrient fluid of the culture. The technique was a modification (by HORVÁTH [5]) of TAKÁTSY's loop method. By means of a 0,2 ml loop, 10^{-1}, 10^{-2}, 10^{-3} etc. dilutions were made of the virus in nutrient fluid. The loop was flamed before each dilution. The inoculated tubes were placed in the horizontal position into an incubator and kept there for 72 hours.

When the cytopathogenic effect of virus was titrated on tissue culture prepared of mouse embryo, the addition of virus was made after 24-hour incubation. Prior to adding the virus inoculum, the tubes not showing signs of tissue growth were discarded. Cultivation was made at 37° C for 72 hours.

The medium used for cultivation in heart tissue of chicken embryo was Simms X_6 solution containing 2,5 per cent embryonic extract. For cultivating mouse tissue, the following nutrient medium was found to be the best: Simms X_6 solution, 87,5 parts; inactivated horse serum, 10 parts; and chicken embryonic extract, 2,5 parts.

Chicken embryo extracts were prepared by homogenizing in a Waring blendor 13-day old chicken embryo torsos with 2 parts of Simms X_6 solution. The homogenizate was centrifuged and stored in the frozen state.

Tissue cultures were checked daily under a hundredfold magnification and the findings were recorded. The results given in the paper are the final, 72-hour values.

Titration for cytopathogenic effect caused no difficulties in chicken heart cultures, since cellular disintegration was highly regular and gradual. The cytopathogenic titre was considered to be the highest dilution of virus causing typical changes in at least 50 per cent ot the explants.

In mouse tissue cultures it rarely occurs that all cells should disintegrate. Under the conditions employed the control cultures (i. e. those not infected with virus) usually remained intact for seven or more days. Thus destruction within 72 hours could be taken as reliable evidence of viral infection. Disintegration of cells started in most cases at the margin of the explant and spread therefrom in the direction of the fibroblast ring. Sometimes it occurred that the culture was completely disintegrated in areas adjacent to the explant, while in marginal areas the cells remained perfectly intact.

Tests for the presence of inclusion bodies. Thin glass plates measuring 60 mm by 10 mm were placed into test tubes. The surface of the plates was covered with a thin layer of plasma, and 4 pieces of tissue were embedded into it. The plasma was then clotted with one drop of embryo extract. After adding 4 ml of the adequate nutrient fluid and the appropriate dilutions of virus, the tubes were incubated for 72 hours. Then the glass plates were removed and placed into methanol for 10 minutes. The fixed preparations were stained with Giemsa's solution, dehydrated with acetone, cleared with xylol and mounted in «Permount».

Titration of viral material in mice or in young chicken. Groups of 4 animals each were inoculated intracerebrally with different dilutions of virus. The volume of single inocula was 0,03 ml. DL 50 was calculated according to REED and MUENCH [7].

Experimental

Cultivation of virus in embryonic mouse tissue

Virus was cultivated in tissue suspension of mouse embryo in six series, started at different points of time. Stock virus was used as starting material. In 3 out of the total of 6 series we carried the virus through 47, 18 and 22 passages, respectively, while in the remaining 3 series only a few passages were made. The details of one of the series are shown in Table I.

Table I

Infectivity of virus passaged in tissue suspension of mouse embryo for chicken tjssue culture and for intracerebrally inoculated mice

Passage No.	Cytopathogenic titre (—log value)	LD50 for the mouse (—log value)
3.	2	N. T.
4.	< 1	N. T.
7.	< 1	N. T.
8.	1	3,20
10.	< 1	3,20
12.	1	4,00
14.	2	4,74
15.	2	4,20
16.	1	4,20
18.	< 1	4,79
19.	1	5,50
23.	2	4,74
24.	2	4,24
25.	2	3,74
26.	1	4,74
27.	1	4,74
28.	2	3,50
30.	1	3,74
33.	2	4,24
47.	2	4,24
Geometric Mean	$10^{-1,25}$	$10^{-4,19}$

Note: N. T. = not tested, a value < 1 is considered 0 when calculating the geometrical mean. « < 1 » indicates that the highest concentration of virus tested (the 1 : 10 dilution) was not cytopathogenic.

Thus, the geometrical mean of the cytopathogenic titres was $10^{-1,25}$ (= 1 : 17 dilution) of virus, while in intracerebral assays in mice the mean DL_{50} was $10^{-4,19}$ (= 1 : 15 500 dilution). This means that the virus was approximately eight thousand times more active when inoculated intracerebrally into mice than in its pathogenic effect on the cells of embryonic chicken tissue.

In the series described and in the course of the other series, 46 different MEC (mouse embryo culture) viral materials were titrated in tissue cultures made of chicken embryo. Of these, 34 were tested for intracerebral infectivity in mice. The results of titrations were distributed as shown in Table II.

Table II

Distribution of MEC virus tested according to infectivity for chicken embryo tissue culture and for mice

Distribution of 46 MEC viral material according to cytopathogenic titre for tissue culture		Distribution of 34 MEC viral material according to intracerebral LD50 values	
$< 10^{-1}$ *	11	$10^{-2.6}$—$10^{-3.0}$	2
10^{-1}	18	$10^{-3.1}$—$10^{-4.0}$	14
10^{-2}	15	$10^{-4.1}$—$10^{-5.0}$	17
10^{3}	2	above 10^{-5}	1
Total :	46	Total :	34

* $< 10^{-1}$ indicates that at the lowest dilution tested (1 : 10) the viral material was non-cytopathegenic.

Calculated for the total number of experiments, the geometrical means were as follows. Cytopathogenic titre $10^{-1,15}$ (1 : 14 dilution) ; DL_{50}, $10^{-3,92}$ (1 : 8318 dilution).

Deviations of this nature never occurred with CEC virus, in the case of which the cytopathogenic titre exceeded the value of mouse LD_{50}. For instance, CEC passages 172 to 219 yielded cytopathogenic titres varying from 10^{-6} to 10^{-8}, while their DL_{50} values for mice were in the range from $10^{-3,5}$ to 10^{-5}. The mouse infecting titres of CEC viruses were usually one or two exponents lower than the cytopathogenic titres.

Modification of virus brought about by changing the tissue for its cultivation

According to the experiments described above, the properties of the virus undergo profound changes on growth in tissue suspension of mouse embryo. The most remarkable property of MEC virus is seen in its low cytopathogenic effect on chicken tissue. The question was whether this modification is only temporary, or of permanent character. In order to elucidate this point a number

of experiments have been carried out. CEC virus was passaged in mouse tissue suspension or, inversely, MEC virus was transferred in chicken tissue suspension.

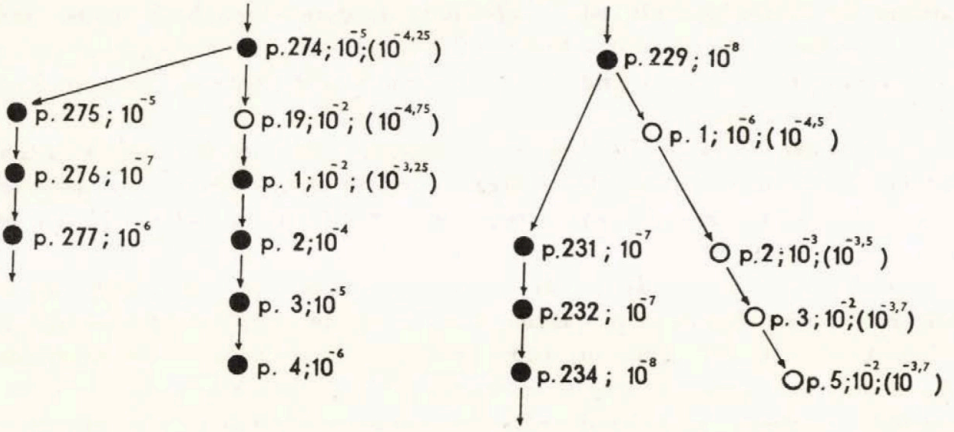

Fig. 1. The variation of cytopathogenic action during serial passages of virus in tissue suspension made either of chicken or mouse embryo

Explanation: ● = tissue suspension of chicken, O = that of mouse embryo. p = number of the passage; the number is representing the cytopahogenic titre for chick en tissue culture; numbers in brackets show the MD_{50} for mouse infected intracerebrally

It is seen that when CEC virus is serially passaged in mouse tissue suspension, the cytopathogenic effect on chicken tissue will decrease considerably after a few passages already. This change, however, did not prove to be permanent, as on passages in chicken tissue suspension the virus has soon regained its former characteristics.

In the experiments described above, passage was made with considerable amounts of inoculum, 0,4 ml of the undiluted homogenizate of 48 or 72 hour cultures. For this reason it had to be assumed that if the particles of CEC virus were not uniform in their properties they would not become homogenous by selection even after a great number of passages. On grounds of this consideration the following experimental method was employed for promoting a potential selection.

CEC virus from the 280th passage, diluted 1 in 1000, was passaged in chicken embryo suspension, using daily transfers. In this case it was not the homogenizate of cultures, but 1 : 1000 dilutions of the nutrient fluid that were transferred. Then CEC virus, obtained after 10 transfers on consecutive days and diluted 1 in 100, was inoculated into tissue suspension of mouse embryo and the homogenizate of the culture was further transferred at 48 hour intervals. The cytopathogenic titre of the first mouse passage was 10^{-4}. The second passage yielded the same value, with the mouse LD_{50} being $10^{-3,5}$. In the fourth passage the cytopathogenic titre was already as low as 10^{-2}. This shows that the method has failed to enhance the stability of CEC virus.

Comparative studies of the properties of CEC and MEC virus

The above described experiments showed that Ay virus grown in tissue suspension of mouse embryo had a very slight pathogenic effect on the cells of chicken embryo. The low cytopathogenic titres of such MEC virus cannot be

ascribed to an inferior growth of virus in mouse tissue suspension. The LD_{50} titres of MEC virus in mouse (average value, $10^{-3,92}$) also appear to point in this direction. This, however, does not truly reflect actual relations and a comparison is acceptable only when infectivity is determined both mouse and chicken embryo tissue cultures. In Table III are shown the cytopathogenic titres of MEC virus for the mouse and for the chicken embryo tissue as determined on 11 different occasions.

As shown in Table III, there was a marked difference between the titres for the two species of animal. The destructive effect of MEC virus on mouse cells exceeds by far the mean of the titres found after intracerebral inoculation of mice ($LD_{50} = 10^{-3,92}$).

It has been reported [3] that Ay virus passaged in tissue suspensions of chicken embryo differs in properties from Ay virus obtained from organs of infected animals. A conspicuous and in many respects characteristic symptom of pseudorabies is the fact that mice inoculated intracerebrally with viral material from naturally or artificially infected mammals succumb very rapidly after

Table III

The cytopathogenic effect of MEC virus on chicken and on mouse tissue cultures

MEC viral material	Cytopathogenic titre	
	for chicken tissue	for mouse tissue
Series III. passage 23.	10^{-2}	10^{-8}
« IV. « 6.	$< 10^{-1}$	10^{-6}
« IV. « 8.	10^{-1}	10^{-8}
« V. « 4.	10^{-2}	10^{-5}
« V. « 8.	$< 10^{-1}$	10^{-5}
« V. « 9.	10^{-1}	10^{-5}
« V. « 10.	10^{-1}	10^{-6}
« V. « 11.	$< 10^{-1}$	10^{-6}
« VI. « 11.	10^{-2}	10^{-4}
« VI. « 13.	$< 10^{-1}$	10^{-5}
« VI. « 17.	10^{-2}	10^{-6}

an incubation period of 3 to 4 days duration, exhibiting acute symptoms frequently associated with severe pruritus. In contrast with this, the latent period of CEC virus infection is somewhat longer, pruritus is relatively rare and the animals die of a gradually developing encephalitis. The pathogenic properties of the two viruses show the same differences. Information as to the incidence of pruritus among mice infected with either of the two types of virus is to be found in Table IV.

Table IV

Incidence of pruritus among mice dead of infection with CEC or MEC virus

Dilution of virus	CEC viral material			MEC viral material		
	Number of animals	Succumbed	Incidence of pruritus, %	Number of animals	Succumbed	Incidence of pruritus, %
10^{-1}	108	108	9,2	108	108	21,3
10^{-2}	108	108	11,0	108	108	22,2
10^{-3}	108	108	2,7	108	105	15,2
10^{-4}	108	70	4,3	108	40	22,5

In mice infected with MEC virus both the duration of the incubation period and the symptoms of disease are similar to the usual pattern developing after inoculation with viral material from infected mammals. After a short latency (usually not longer than 56 to 70 hours) the animals develop acute symptoms and die. Mad itch, a symptom of severe pruritus, is frequently seen.

In earlier studies [3] it was found that the susceptibility to Ay virus of young chicks is comparable to that of albino mice. The conditions were identical for virus obtained from cultures in tissue suspension of chicken embryo or for virus from the brain of intracerebrally inoculated rabbits. As it is shown in Table V, CEC and MEC virus are almost equally pathogenic for mice and for chickens inoculated intracerebrally. This means that the low destructive effect of MEC virus on chicken embryo tissue cultures is not proportionate to the high infectivity for intracerebrally inoculated chickens.

Table V

Pathogenicity of CEC and MEC virus for chicken and mice, as determined by intracerebral inoculation

Dilution of virus	CEC virus: 290th passage Cytopathogenic titre for chick tissue, 10^{-6} for mouse tissue, 10^{-3}		MEC virus, 15th passage Cytopathogenic titre for chick tissue, 10^{-1} for mouse tissue, 10^{-5}	
	chicken	mouse	chicken	mouse
10^{-1}	4/4	4/4	4/4	4/4
10^{-2}	4/4	4/4	4/4	4/4
10^{-3}	4/4	3/4	4/4	4/4
10^{-4}	4/4	2/4	1/4	1/4
10^{-5}	3/4	0/4	0/4	0/4
10^{-6}	0/2			

Note: The numerator shows the number of dead animals, the denominator shows the number of animals inoculated.

Interference between MEC and CEC virus

The fact that MEC virus had a mild pathogenic effect on chicken tissue cultures is suggesting that under adequate conditions the virus might interfere with CEC virus and would thus be able to modify the high destructivity of the latter in tissue cultures of chicken embryo. Before all, it had be elucidated wether MEC virus would actually multiply in chicken explants inoculated with high dilutions of virus, i. e. where no cytopathogenic effect is visible. The following experiment was carried out.

To a set of test tubes each containing 8 pieces of embryonic chicken heart embedded in plasma a serial dilution of MEC virus in nutrient fluid was added and incubated for 72 hours. Control tubes containing only virus dilution is nutrient fluid were also incubated to check the thermoinactivation of virus. After incubation the cultures were throughly examined in 100 fold magnification for determining eventual cytopathogenic effects. Finally, the content of each tube was homogenized and the suspension was inoculated intracerebrally into 3 mice. Fluid from the control tubes was also tested for infectivity. The results are summarized in Table VI.

Table VI

Intracerebral mouse infectivity of chicken heart cultures infected with MEC virus. 72-hour values

Dilution of MEC virus	Chicken heart cultures		Control viral material. Infectivity
	Cytopathogenic effect	Infectivity	
10^{-1}	+	N. T.	0/3**
10^{-2}	±*	3/3**	0/3
10^{-3}	—	3/3	0/3
10^{-4}	—	2/3	0/3

* In 2 out of the 8 explants destruction was definitely detectable at 72 hours.
** Death rate in mice inoculated with tissue culture and control virus, respectively.

It can be read from Table VI that the presence of virus was demonstrable also in cultures not exhibiting cytologic changes. In the control tubes by the end of the cultivation period the virus has lost its infectivity. These findings confirm the view that the absence of changes indicative of cytopathogenic effect does not yet indicate that multiplication or viability of MEC virus has ceased.

In some similar experiments quantitative relations were also considered. It was found that a 1 to 10 dilution of the homogenizate from a chicken heart culture, inoculated previously with 1 to 1000 dilution of MEC virus, is still pathogenic for mice. This means a culture infectivity titre of 1 to 2000, as related to the quantity of minced tissue. Accordingly, in cultures not showing

Table VII

Interference of MEC virus, as determined by action on the cytopathogenic effect of CEC virus
(Heart tissue cultures from chick embryo)

Experiment No.	Minced chick heart treated with MEC virus			Control (nott reated)
	Duration of treatment	Dilution of MEC virus	Cytopathogenic titre of CEC virus	Cytopathogenic titre of CEC virus
1.	8h	1 : 1000	10^{-8}	10^{-8}
2.	18h	1 : 1000	10^{-5}	10^{-8}
	18h	1 : 10000	10^{-5}	10^{-8}
3.	21h	1 : 1000	10^{-3}	10^{-8}
4.	21h	1 : 1000	10^{-4}	10^{-8}
5.	20h	1 : 100	10^{-3}	10^{-7}
	20h	1 : 1000	10^{-4}	10^{-7}
6.	20h	1 : 100	10^{-3}	10^{-7}
	20h	1 : 1000	10^{-3}	10^{-7}
7.	18h	1 : 100	10^{-4}	10^{-7}
8.	20h	1 : 100	10^{-4}	10^{-7}
	20h	1 : 1000	10^{-3}	10^{-7}

Fig. 2. Appearance of tissue culture of chicken heart incubated with 10^{-4} dilutions of MEC virus for 24 hours and infected with 10^{-5} CEC virus. Magnification, ×120

signs of cytopathogenic action the virus may not only survive but actually multiply. Further support to this view was lent by the results of interference experiments, in which the following technique was used.

To 18 ml of Simms X_6 solution in a 100 ml Erlenmeyer flask 40 to 50 fragments obtained of a pool of embryonic chicken hearts were added and inoculated

with 2 ml of the adequate viral dilution (MEC virus). Control not containing virus was also provided for. The flasks were kept in an incubator for 8 to 24 hours, then explants embedded in plasma were prepared from each flask and the cultures thus obtained were inoculated with serial dilutions of CEC virus.

Fig. 3. High power view of specimen shown in Fig. 2. Magnification, ×360

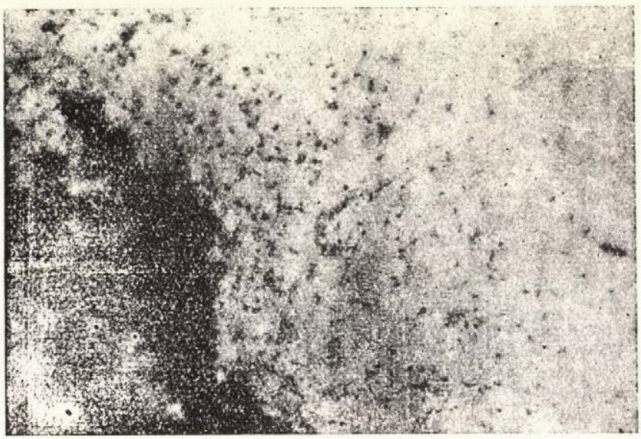

Fig. 4. Chicken embryo heart culture infected with 10^{-7} CEC virus. After complete disintegration of cells, remnants of nuclei are visible at sites. Magnification, ×120

After 72 hours of cultivation the limiting dilution initiating cytopathogenic changes was determined. Of course, care was taken to detect an eventual cytopathogenic action exerted by the dilution of MEC virus employed. No such activity was observed on the experiments described.

As seen, pieces of chick embryo heart kept in contact with dilute MEC virus for 18 to 24 hours have lost much of their susceptibility to CEC virus. In tissue cultures showing interference the growth of explants was comparable in degree to that in cultures not infected with virus. Examination of the cultures in the native state at a 100 fold magnification revealed no changes pointing to cell injury.

The interference phenomenon was not stationary in the sense that the 72-hour results would have persisted later. On continued cultivation the rate of cell destruction gradually increased in proportion with the concentration of CEC virus. The detailed morphological description of the cultures will be omitted. Instead of this, a few figures are presented to show the typical changes observed in the course of our studies on the interference phenomenon.

Interference between the viruses could be demonstrated also by a different technique. In tissue cultures prepared from the heart of chicken embryo by embedding the fragments in plasma and inoculating with 10^{-3} MEC viral material, no cytopathogenic effect was observable. 24 hours later the nutrient fluid was removed and serial dilutions of CEC virus in the new nutrient fluid were added to the tubes. The cytopathogenic titre of MEC virus cultures was 10^{-4}, as compared with the 10^{-7} titre yielded by titre the controls which had not been treated with MEC virus.

Examinations for the presence of inclusion bodies

In earlier studies we had failed to detect the presence of inclusion bodies in the cells [1] of chicken embryo tissue cultures infected with the same strain of Ay virus which has been employed in the present experiments. This finding, which is not in accordance with our knowledge of Ay virus, has been rechecked in a great number of tissue cultures prepared under various conditions. Explants from chicken heart and from mixed tissues of mouse embryo were embedded in either chick or rabbit plasma, and the cultures were inoculated with CEC as well as MEC viral material, in dilutions of from 10^{-2} to 10^{-6}. Cultures which after cultivation for 72 hours still contained intact cells were stained with Giemsa's solution and examined for presence of inclusion bodies. A considerable number of tissue cultures prepared by different methods as outlined above has been examined microscopically, but inclusion bodies could not be detected in any of them. Necrobiosis of cells was never associated with the formation of inclusion bodies.

Discussion

The multiplication in explanted surviving tissue or in tissue cultures of most viruses causing disease in mammals can be detected by their injuring effect on cells. With or without the appearence of inclusion bodies the cells gradually

degenerate, or, more frequently, become completely destroyed. The simple technique had stimulated IVÁNOVICS and HYDE [8] as early as 1936 to titrate infectivity of rabbit virus III *in vitro*, by means of tissue cultures. Tissue cultures are now important aids in quantitative virus research. Although the problem has aroused considerable interest, our knowledge concerning growth in tissue cultures, cytopathogenic effect, pathogenicity for animals etc., of certain viruses is still very meagre. The present study on the Ay virus contributes new evidence to these problems.

In the course of our experiments, a strain of Ay virus has been carried through several hundred passages in tissue suspension of chick embryo. The viral material so maintained differs somewhat from the virus recoverable from naturally or artifically infected mammals. The difference manifests itself also in mice infected intracerebrally. Animals infected with virus derived from tissue suspension of chicken embryo succumb with symptoms less characteristic of Ay disease than those displayed by animals inoculated with organ extracts from infected animals. So, for instance, pruritus rearely occurs in the former group. The properties of virus obtained in chicken embryo culture (CEC virus) are not firmly established and may be reversed easily, e. g. after a single intracerebral mouse passage, or by growth in tissue suspension of mouse embryo. Even viral material that had been passaged almost 300 times in chicken embryo cultures obtained new properties after a few passages in mouse tissue (MEC virus).

The most remarkable property of MEC virus is its weak cytopathogenic effect on chicken embryo tissue. In contrast with this it is highly pathogenic for the cells of mouse embryo. As the Ay virus material is still cytopathogenic at dilutions higher than those capable of causing infection in mice, it appears that larger numbers of viral particles are required for causing lethal infections in the mouse than infect mouse cells under *in vitro* conditions. This fact, along with the high variability of the cytopathogenic effect of Ay virus are apparently in support of our hypothesis on the cyclic type of growth of virus multiplying in the excised chorio allantoic membrane [4]. At any rate, the present experiments support our earlier assumption as to the high heterogeneity of Ay virus populations.

Our observations have contributed new data in support of the view that the cytopathogenic effect is independent, or at least partly independent, of the multiplication rate of virus. As early as 1949, BANG and GEY [19] reported that the destruction of cells in tissue cultures infected with the virus of Eastern equine encephalomyelitis is not parallel with the number of viral particles formed in cells. SABIN, WINSSER and HENNESEN [10] observed that intracerebral passage of poliomyelitis virus in monkeys segregated a variant which was ineffective in destructing monkey fibroblasts, but exerted a strong cytopathogenic activity on epithelial cells. The recent observation of ACKERMANN, ROBSON and KURTZ [11] is remarkable. These authors have found

that in HeLa cultures infected with the Saukett strain of poliomyelitis virus treatment with p-fluorophenylalanine resulted in an absence of virus multiplication. At the same time, the cytopathogenic effect of the virus was unchanged. HENLE, GIRARDI and HENLE [12] have reported that in HeLa cell cultures influenza virus was highly destructive, but no infective virus was produced. Infection of HeLa cells led only to formation of an incomplete haemagglutinating viral material. The observations on Ay virus reported in the present paper are in harmony with the above data, insofar as with this pathogenic agent, too, the properties of the viral material are profoundly influenced by the host cells in which the virus is multiplying.

It is remarkable that MEC virus can antagonize the destructive effect of CEC virus. A tissue culture infected with the slightly pathogenic variant may thus be protected against the highly destructive virus. This observation of ours is similar to that made recently by SABIN [13], who has found that a strain of suckling mouse-adapted poliomyelitis MEF_1 virus exerted little or no cytopathogenic effect on monkey kidney cells. Monkey tissue cultures inoculated with such viral material proved to be resistant to the original, highly cytopathogenic virus, and also that the MV and Y—SK strains of poliomyelitis virus show interference.

It is difficult to explain why we have failed to demonstrate the presence of inclusion bodies in explanted embryonic chicken or mouse tissue infected with Ay virus. Inclusion bodies in tissues infected with Ay virus have been observed by several authors in rabbit testicle cultures [14, 15], in pure fibroblast cultures obtained from mice [16] and, more recently, in HeLa cells. The virus used in these studies was almost without exception the Aujeszky's original strain. We cannot explain why we did not detect inclusion bodies in the course of our experiments. Formation of inclusion bodies is not a *sine qua non* of Ay virus infection; HIRT [18] found no inclusion bodies in the organs of naturally infected pigs. Our negative results could not be due to the exclusive use of chicken tissue, since in the experiments described mouse tissue cultures were also employed. On the other hand, inclusion bodies have already been described in the chicken embryo infected with pseudorabies virus [19].

The most plausible explanation of the absence of inclusion bodies appears to be that individual strains of the pseudorabies virus may differ in inclusion body production. The problem will have to be elucidated in further studies.

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ВАРИАЦИЯ ЦИТОПАТОГЕННОГО ДЕЙСТВИЯ ВИРУСА PSEUDORABIES (БОЛЕЗНЬ АУЕССКОГО)

Г. ИВАНОВИЧ, И. БЕЛАДИ и Э. СЁЛЛЁШИ

Резюме

1. Авторам удалось сохранить вирус Pseudorabies (болезнь Ауесского) более, чем в 300 пассажах в тканевой взвеси куриного зародыша. Культивированный таким образом вирус является весьма деструктивным в отношении клеток куриных тканевых культур и, кроме того, в некоторой степени изменяется его патогенность для мышей, исследуемая путем внутримозговой прививки. Свойства адаптированного вируса весьма быстро изменяются после нескольких пересевов на взвесь ткани мышиного зародыша.

2. Наиболее бросающимся в глаза свойством вирусного материала, сохраняемого в тканевой культуре мышиного зародыша, является резкое понижение деструктивного действия на клетки куриного зародыша. Вирусный материал мышиной тканевой культуры вообще в 10 000 100 000 раз более цитопатогенен для мышиных, чем для куриных клеток. Разница эта однако является выраженной только в тканевых культурах, но совсем незначительна при определении заразительности материала на мышах и молодых цыплятах путем внутримозговой прививки.

3. Тканевые культуры куриного сердца, содержимые в контакте с вирусным материалом, адаптированным к мышиным тканевым культурам, становятся в значительной степени резистентными к обладающему высокой цитопатогенностью куриному вирусу. Таким образом видоизменившийся в мышиной тканевой культуре вирус выявляет феномен интерференции против цитопатогенного действия куриного вируса.

4. Описанные свойства вируса, адаптированного к мышиным тканевым культурам непостоянны. Совершенно изменяются после нескольких пассажей в куриных тканевых культурах. Повидимому, популяции вируса Ауесского являются весьма гетерогенными и условия размножения в большой степени влияют на их состав.

5. Изученный штамм не образует внутриядерных включений ни при одном из самых различных условий культивирования в тканевых культурах, ни мышиных, ни куриных зародышей. Это указывает на существование различия между отдельными штаммами Pseudorabies в отношении образования внутриядерных включений, т. е. это цитопатогенное свойство изменяется и по качеству, соответственно отдельным штаммам.

ZUSAMMENHANG ZWISCHEN DER TEMPERATUR UND WIRKUNG EINZELNER DESINFIZIENZIEN

Von

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Der Zusammenhang zwischen der Wirkung eines Desinfiziens und der Temperatur ist seit langem bekannt. CHICK [1], NYMAN und MADSEN [2] hatten darauf schon früher hingewiesen. Die vorliegende Untersuchung dieses Zusammenhanges war deshalb notwendig geworden, weil bei den unter gleichen Versuchsbedingungen vorgenommenen Serienuntersuchungen neuer Desinfizienzien (quaternärer Verbindungen) [3, 4] abweichende Resultate ergaben und diese nur auf Temperaturdifferenzen zurückgeführt werden konnten. Es schien notwendig, die Zusammenhänge zwischen der Temperatur und der bakteriziden Wirkung nicht nur bei den neuen, sondern auch bei einigen seit langem bewährten Desinfizienzien durch Vergleich zu klären.

CHICK hatte bereits 1908 die bakterizide Wirkung von Phenol, Sublimat und Silbernitrat auf Paratyphusbakterien untersucht. NYMAN und MADSEN prüften die sporizide Wirkung von Sublimat auf Anthraxsporen bei verschiedenen Temperaturen. Die Richtigkeit des von CHICK bei der Berechnung des Temperaturquotienten angewandten Verfahrens wurde von mehreren Autoren bestätigt. In den letzten Jahren (1953) wies BRAUSS [5] darauf hin, dass die widersprechenden Resultate, die sich bei der Untersuchung gleicher Desinfizienzien von seiten mehrerer Forscher ergaben, auf die Durchführung der Untersuchungen bei verschiedenen Temperaturen zurückzuführen waren. Obwohl KOCH [6], BEHRING [7], HAIDER [8] und andere diesbezüglich umfassende Untersuchungen vorgenommen hatten, hielt es BRAUSS doch für notwendig, bei neuen Desinfektionsmitteln (quaternären Verbindungen, Ampholitseifen) die Bedeutung des Temperaturfaktors zu untersuchen. Die moderne Kontrolle der Desinfizienzien erfordert demnach eine Überprüfung unserer bisherigen Untersuchungsmethoden und die Ausarbeitung einheitlicher neuer Verfahren, mit deren Hilfe wir die Wirkung der Desinfektionsmittel in der Praxis beurteilen können.

In vorliegender Arbeit wird lediglich die Bedeutung des Temperaturfaktors behandelt.

Methoden

Unter den zahlreichen Faktoren, deren Veränderung in der Desinfektionswirkung eine entscheidende Rolle spielt, sind folgende am wichtigsten: die Menge der zur Untersuchung verwendeten Bakterien, die Konzentration des Desinfiziens, Einwirkungszeit und Temperatur. In unseren Versuchen fixierten wir bei Veränderung der Temperatur die ersten zwei Faktoren. Die Versuche wurden mit 0,5 ml einer 18—20stündigen Bouillonkultur von Salmonella typhi vorgenommen. Die Keimzahl wurde in der Weise stabilisiert, dass wir den untersuchten Stamm vom Schrägagar in Fleischextraktbouillon (genau festgestellte Menge 12 ml) einimpften, von hier zwecks Erzielung einer annähernd gleichen Keimzahl nach 24 Stunden mit einer Öse von 4 mm Durchmesser in eine neue Bouillon überimpften und dies nach 48 Stunden wiederholten. Von der 18—20stündigen Kultur dieser letzten Umimpfung benutzten wir gleiche Mengen zu den Untersuchungen.

Auf Grund der Ergebnisse früherer experimenteller Untersuchungen (die wir hier nicht im einzelnen behandeln wollen) verwendeten wir als Subkulturen 1% Dextrose enthaltende Fleischextraktbouillon. Im allgemeinen ist man der Auffassung, dass das beste Resultat zu erzielen ist, wenn auch die letzten Spuren des Desinfiziens aus den Subkulturen entfernt werden. Dies geschieht nach ZEISLER am einfachsten in der Weise, dass man in den Nährboden Organstückchen (Leber) legt. Wir vermieden die Hemmungswirkung des Desinfiziens durch Vermehrung der Bouillonmenge. Die bakterizide Wirkung von Formalin kam auch in den 12 ml Bouillon dann am besten zur Geltung, wenn die Versuche statt mit der üblichen 0,1 ml TAKÁTSY-schen Spiralöse mit einer Öse von 4 mm Durchmesser durchgeführt wurden.

Experimenteller Teil

Bei den Untersuchungen wurden 6 Desinfizienzien verglichen: Formalin, $HgCl_2$, Ryfen (Merfen, Quecksilberphenylborat), Phenol, Cetavlon (Cetyltrimethyl-ammoniumbromid) und Nitrogenol (Hexadecylpyridiniumbromid). Die Konzentrationen der Desinfektionsmittel wurden so gewählt, dass wir jene stärksten Verdünnungen bei verschiedener Temperatur (20—25—30—35° C) einstellten, welche 0,5 ml der *Salmonella typhi*-Kultur — zu 4,5 ml Verdünnung des Desinfiziens gegeben — bei 15° C innerhalb 1 Stunde noch nicht, aber innerhalb von 2 Stunden sicher töteten. Wenn z. B. die mehrere Verdünnungen eines Desinfiziens ebenfalls ein ähnliches Resultat ergaben, beispielsweise die Verdünnungen 1 : 120, 1 : 140 von Formalin, so stellten wir die stärkste Verdünnung ein, d. h. 1 : 140. Auf diese Weise wurden, wie aus Tabelle I hervorgeht, Des-

Tabelle I

Wirksame Konzentrationen bei 15° C 2 Stunden hindurch verschiedener Desinfizienzien
(verwendetes Bakterium: S. typhi)

Desinfizienzient	Wirksame Konzentration
Formalin	1 : 140
$HgCl_2$	1 : 180 000
Ryfen	1 : 40 000
Phenol	1 : 115
Cetavlon	1 : 11 000
Nitrogenol	1 : 12 000

infizienzien von verschiedener Konzentration, aber gleicher Wirkung miteinander verglichen.

Die Einwirkung erfolgte bei 20—25° C 40 Minuten lang in Abstufungen von 5 Minuten, später in Abstufungen von 10 Minuten (50—60—70—80—90). Bei 35° C wurden die Versuche auch noch mit einer Einwirkungszeit von 0,5, 1,3 und 7 Minuten eingestellt. Bei jeder Temperatur und Einwirkungszeit wurden im allgemeinen 10 (bei Sublimat nur 6) Untersuchungen vorgenommen. Hierbei stellten wir die Einwirkungszeit fest, die zur Vernichtung der Bakterien bereits mit Sicherheit genügte. Als Beispiel zeigen wir die mit der Phenolverdünnung 1 : 115 bei 20° C durchgeführten, auf Salmonella typhi bezüglichen Resultate (Tabelle II).

Tabelle II

Experimentelles Resultat mit der Phenolverdünnung 1 : 115 bei 20° C
(verwendetes Bakterium: S. typhi)

Bezeichnung der Parallelunter-suchung	Einwirkungszeit in Minuten													Erfolgreiche Einwirkungszeit in Minuten
	5	10	15	20	25	30	35	40	50	60	70	80	90	
I	+	+	+	+	+	+	+	+	+	+	+	—	—	80
II	+	+	+	+	+	+	+	+	+	+	+	—	—	80
III	+	+	+	+	+	+	+	+	+	+	+	+	—	90
IV	+	+	+	+	+	+	+	+	+	+	+	—	—	80
V	+	+	+	+	+	+	+	+	+	+	+	—	—	80
VI	+	+	+	+	+	+	+	+	+	+	+	—	—	80
VII	+	+	+	+	+	+	+	+	+	+	—	—	—	70
VIII	+	+	+	+	+	+	+	+	+	—	—	—	—	60
IX	+	+	+	+	+	+	+	+	+	—	—	—	—	60
X	+	+	+	+	+	+	—	+	—	+	+	—	—	80

Die letzte Kolonne der Tabelle gibt die bei den einzelnen Paralleluntersuchungen gewonnenen wirksamen Einwirkungszeiten an.

Die auf je 1 Temperaturgrad bezüglichen geometrischen Durchschnittszahlen der Resultate der erhaltenen Einwirkungszeit sind auf Tabelle III wiedergegeben.

Aus den Durchschnittswerten der Tabelle kann festgestellt werden, dass bei sämtlichen untersuchten Desinfektionsmitteln zwischen Temperatur und Einwirkungszeit ein entschiedener Zusammenhang vorliegt. Dieser Zusammenhang besteht darin, dass bei höherer Temperatur zur Abtötung der Bakterien eine kürzere Zeit benötigt wird. Diese hochgradige Zunahme der Reaktionsgeschwindigkeit entspricht den bei chemischen Reaktionen beobachteten ähnlichen

Tabelle III

Durchschnittliche Einwirkungszeit der Desinfizienzien in Minuten bei verschiedenen Temperaturen (S. typhi)

Desinfiziens	20°	25°	30°	35°
Formalin	59,2	41,7	22,4	15,1
$HgCl_2$	—	54,1	21,5	16,3
Ryfen	46,0	30,5	10,8	6,5
Phenol	75,2	27,4	11,5	4,8
Cetavlon	—	9,6	3,7	0,9
Nitrogenol	—	18,3	6,8	1,6

Erscheinungen. Das Phänomen lässt sich vielleicht einerseits damit erklären dass die Empfindlichkeit (Permeabilität?) der Bakterien gegenüber den Desinfektionsmitteln mit der Erhöhung der Temperatur zunimmt, anderseits die Reaktionsgeschwindigkeit zwischen den Molekülen des Desinfiziens und den empfänglichen Bestandteilen der Bakterien ansteigt.

CHICK stellte bei seinen mit 0,6%igem Phenol an Salmonella durchgeführten Versuchen fest, dass für den Desinfizierungsprozess folgender exponentieller Zusammenhang gilt:

$\frac{k'}{k} = \theta^{(T'-T)}$, wobei k bzw. k' die die Reaktionsgeschwindigkeit kennzeichnenden Konstanten bei T' bzw. T Temperatur bedeuten, während θ die für das Desinfiziens und die verwendeten Bakterien charakteristische konstante Zahl darstellt, die wir als *Temperaturquotienten* bezeichnen. Wir wissen, obwohl wir dies bei unseren Reaktionsgeschwindigkeitsuntersuchungen nicht feststellen konnten, dass Reaktionsgeschwindigkeit und Einwirkungszeit im umgekehrten Verhältnis zueinander stehen. Deshalb lassen sich bei der Bestimmung des Temperaturquotienten die Angaben der Einwirkungszeit ohne weiteres verwenden. Setzen wir in obige Gleichung die Angaben der Einwirkungszeiten ein, so erhalten wir: $\frac{t}{t'} = \theta^{(T'-T)}$, logarithmiert und geordnet: $\lg t = \lg t' + + T' \lg \theta - T \lg \theta$, wobei $\lg t' + T' \lg \theta$ konstant ist, $\lg t$ der Logarithmus der Einwirkungszeit, der eine lineare Funktion der Temperatur T darstellt, Richtungstangente: $-\lg \theta$. Im folgenden bestimmten wir dementsprechend unter Zugrundelegung der Logarithmen der Einwirkungszeit unter Anwendung der Methode der kleinsten Quadrate je Desinfektionsmittel die den Zusammenhang zwischen Temperatur und Einwirkungszeit zum Ausdruck bringenden Geraden. Auf der Zeichnung (Abb.1) demonstrieren wir als Beispiel die Berechnungen, die auf Grund der Ergebnisse der Phenolverdünnung 1 : 115

auf Salmonella typhi vorgenommen wurden. Auf der rechten Seite der Zeichnung ist die Gerade dargestellt, die den Zusammenhang zwischen den Logarithmen der Einwirkungszeit und der Temperatur bestimmt. Die mit o bezeichneten Werte sind die Durchschnittswerte der festgestellten Untersuchungsresultate bei einer gegebenen Temperatur; diesen Punkten passt sich die Regressionsgerade gut an. Es sei bemerkt, dass nach unseren Kontrollberechnungen der geradlinige Zusammenhang bei allen Desinfizienzien statistisch bestätigt werden konnte.

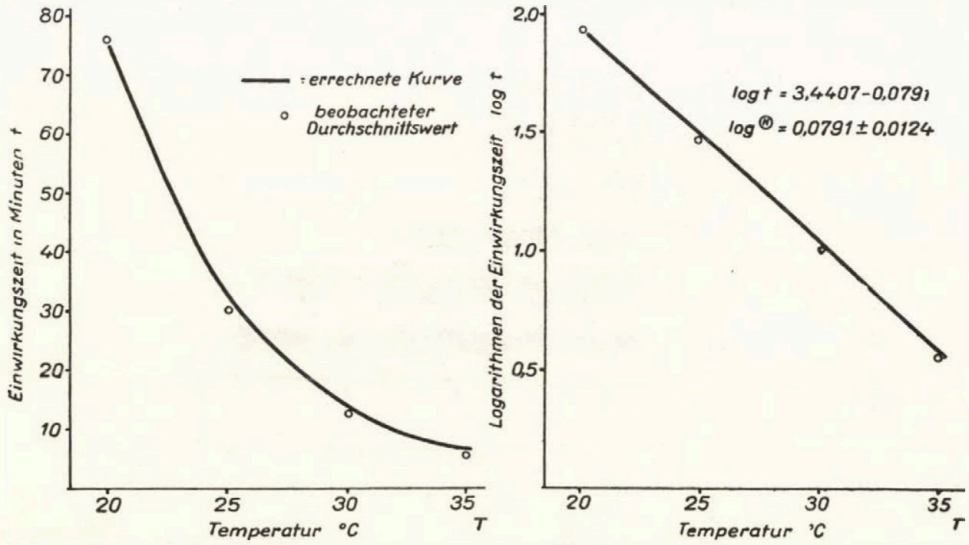

Abb. 1. Zusammenhang zwischen durchschnittlicher Desinfizierungszeit und Temperatur (S. typhi; Phenol 1 : 115)

Dies bedeutet, dass die vorhin angegebene Gleichung für die Zusammenhänge zwischen Einwirkungszeit und Temperatur auch bei unseren Untersuchungsmethoden als gültig betrachtet werden kann. Wenn wir anstelle der Logarithmen der Einwirkungszeit deren Numeri anwenden, bekommen wir die auf der Abbildung links angeführte absteigende exponentielle Kurve, der sich die im Laufe der Versuche erhaltenen Werte naturgemäss ebenso anpassen wie auf der vorigen Abbildung. Dieser exponentielle Zusammenhang besagt, dass bei jeder beliebigen Temperatur die gleich grosse Erhöhung der Temperatur die Einwirkungszeit in stets gleichem Verhältnis verkürzt. Wir bemerken, dass wir das am Beispiel des Phenols veranschaulichte Verfahren auch bei den übrigen Desinfizienzien zusammen mit der erforderlichen statistischen Kontrolle durchgeführt haben. (Die exponentielle Kurven und geradlinige Zusammenhänge aufweisenden Regressionsgeraden bringen wir wegen Raummangel nicht zur Darstellung.)

Abb. 2 zeigt den Wert der bei den untersuchten Desinfizienzien festgestellten Temperaturquotienten. Diese wurden zwecks besserer Bewertung der Grössenordnungen auf 10° C Temperaturveränderung umgerechnet. Dies bedeutet, dass z. B. bei Cetavlon parallel mit der Erhöhung der Temperatur um 10° C die Reaktionsgeschwindigkeit auf das 10,6fache zunimmt oder, was von gleicher Bedeutung ist, dass die erforderliche Einwirkungszeit auf den 10,6ten Teil sinkt. Umgekehrt wiederum bedeutet es, dass bei der um 10° C niedrigeren Temperatur die Reaktionsgeschwindigkeit in diesem Verhältnis abnimmt und daher im Interesse einer erfolgreichen Durchführung der Desinfektion die Einwirkungszeit

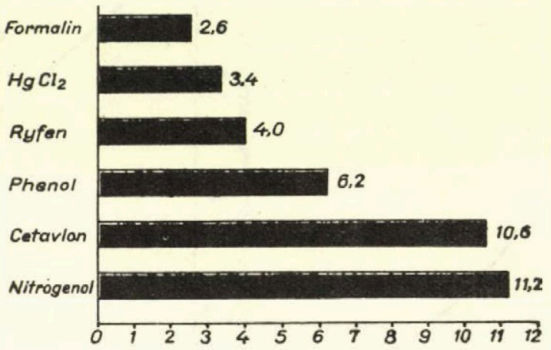

Abb. 2. Wert der Temperaturquotienten je 10° C

auf das 10,6fache erhöht werden muss. In diesem Zusammenhang muss auch in Betracht gezogen werden, dass die Veränderung der Konzentration des Desinfektionsmittels jeweils eine andere Wirkung herbeiführt.

Auf Grund der errechneten Temperaturquotienten sind die untersuchten Desinfizienzien folgendermassen zu bewerten. Am wenigsten empfindlich reagiert auf Temperaturveränderungen Formalin, dessen desinfizierende Wirkung auf Salmonella typhi sich je 10 Grad um das 2,6fache erhöht. Zwischen der 3,4fachen Wirkungssteigerung des $HgCl_2$ und der 4,0fachen des Ryfen besteht kein grosser Unterschied. In der Grössenordnung folgen hiernach die Temperaturquotienten von Phenol mit 6,2, der quaternären Verbindungen Cetavlon mit 10,6 und Nitrogenol mit 11,2. Unsere Ergebnisse stehen im Einklang mit den Literaturangaben, laut welchen der Temperaturquotient des Phenols etwa 7—8 beträgt, der von $HgCl_2$ hingegen auf je 10 Grad berechnet auf 2—4 fällt. Die Hitzeempfindlichkeit der quaternären Verbindungen ist allgemein bekannt [9].

Zusammenfassung

1. Zwischen der Wirkung eines Desinfiziens und der Temperatur besteht ein entschiedener Zusammenhang. 2. Die Untersuchungen der Desinfizienzien können zwar bei Zimmertemperatur vorgenommen werden, doch ist ihr Temperaturquotient unbedingt zu bestimmen und die Gebrauchsanweisung unter Berücksichtigung der mit der Temperaturveränderung verknüpften Wirkungsabnahme oder -zunahme anzugeben. 3. Eine neue biologische Methode zur Bestimmung des Temperaturquotienten wurde ausgearbeitet. 4. Ein bei jeder Temperatur gleichmässig wirksames Desinfizienz konnte nicht gefunden werden.

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ЗАВИСИМОСТЬ МЕЖДУ ТЕМПЕРАТУРОЙ И ДЕЙСТВИЕМ НЕКОТОРЫХ ДЕЗИНФИЦИРУЮЩИХ СРЕДСТВ

Й. СИТА и ДЬ. БАРШИ

Резюме

1. Существует определенная связь между действием дезинфицирующих средств и температурой. 2. Исследования дезинфекционных средств можно производить при комнатной температуре, но в дальнейшем обязательно необходимо определять их температурный коэффициент и давать практическую инструкцию, принимая во внимание повышение или понижение эффективности в зависимости от изменения температуры. 3. Авторы разработали новый метод определения температурных коэффициентов. 4. Не обнаружили идеального дезинфицирующего средства, обладающего одинаковой эффективностью при различной температуре.

A RAPID METHOD FOR DETERMINING ANTIBIOTICS IN A SINGLE DROP OF FERMENTATION FLUID

By

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Great numbers of shaker cultures must be examined for finding high antibiotic producer strains and for selecting the most suitable medium. In the laboratory this can be carried out in micro volumes and so that the quantity of test samples is rather limited. A combination of the paper disc method (which had been used for determining microbial sensitivity first [1—4] and was only later adapted for quantitative work [5—6]) with TAKÁTSY's spiral loop technique recently introduced in serology [7—8] has made it possible to test a great number of samples in small single volumes.

The paper disc method, apart from being rapid has the advantage of having a wide range of the assay and thus one, or, at the most, two, dilutions suffice for the determination of the concentration of the antibiotic at kind of any fermentation.

The method to be described was developed for the purpose of streptomycin determination in the following form.

To a soy bean medium (composed of cornsteep, 6,0 g ; soy bean meal, 20 g ; dextrose, 20,0 g ; sodium chloride, 3,0 g ; Na_2CO_3, 2,0 g ; tap water 1 liter, pH 7,2, autoclaved at 2,0 atm. for 20 minutes) was added a solution of streptomycin sulphate of known concentration and tested for maltol reaction in a Beckman DU spectrophotometer before use, so that the concentration of streptomycin in the medium was 640 μg. In preliminary tests it was established that under the conditions employed the medium did not bind streptomycin so that it was possible to recover the total amount of streptomycin which had been added to it. A 0,1 ml spiral loop was burned off and the sample taken with it from the medium was rotated in 2,5 ml of a pH 8,0 phosphate buffer, thus making a 1 : 26 dilution, the solution being diluted to 24,6 μg/ml. Using a 0,025 ml loop, a sample of this dilute solution was transferred to a filter paper disc 12 mm in diameter which was then placed on a pH 8 nutrient agar plate (broth, 1,0 litre ; peptone, 0,5 per cent ; agar, 1,8 per cent ; autoclaved at 1,5 atm.) containing *B. subtilis* (ATCC 6633) spores in adequate concentration. Plates 17,5 cm by 25,5 cm in size were used in the titrations. The calibration curves were determined with standard solutions, 10, 25, and 60 μg in concentration, respectively. (Table I)

Table I

Date (Plate No.)	Size of zone inhibition in mm	Average size, mm and the sigma	Streptomycin μg/ml		Percentage of difference between calculated and measured concentration
			calculated	measured	
1955. III. 9. (1)	25,2, 26,4, 25,3, 25,0	25,4 ± 0,63		26,3	+ 6,9
	24,8, 25,5	25,15 ± 0,49		23,1	— 5,7
	25,5, 25,5, 25,2, 25,5	25,4 ± 0,15		26,4	+ 8,3
(2)	24,9, 25,2, 26,0, 25,9	25,5 ± 0,52		27,3	+ 12,0
	25,6, 25,0	25,3 ± 0,42		25,4	+ 3,2
(3)	25,5, 25,8, 25,8, 25,2	25,5 ± 0,30		23,8	— 3,3
	25,2, 25,6	25,4 ± 0,28	24,6	23,3	— 5,2
III. 28. (4)	24,4 24,2, 24,9, 24,5	24,4 ± 0,31			
	24,0, 24,5, 24,2, 24,8			25,0	+ 1,6
	22,2, 22,4, 22,5, 21,5	22,3 ± 0,40		25,8	+ 4,9
	22,5, 22,2, 22,8				
(5)	22,0, 22,3, 22,0, 22,0	22,07 ± 0,15		23,9	— 2,8
	21,9, 22,0, 22,2, 21,8	22,02 ± 0,20		23,5	— 4,4
	22,0, 22,2, 21,8, 22,0	22,0 ± 0,16		23,5	— 4,4

The precision of the method is comparable to that of the diffusion techniques in common use.

The filter paper discs were cut from Macharey Nagel No. 214 filter paper by means of an apparatus devised for this purpose. The water absorbing capacity of the paper used was adequate for this type of work [9].

The filter papers were kept in an exsiccator for 24 hours prior to use. Since the paper disc method allows to estimate streptomycin in the concentration range from 2,5 to 60 μg/ml, by using the above 1 : 26 dilution the range of determinable concentrations varied from 65 to 1560 μg/ml. If it is desirable to measure concentrations higher than those mentioned, the following procedure may prove useful. The dilution is made in a larger volume of buffer or a second dilution is made by taking with the loop 0,025 ml from the first dilution and rotating it in 0,25 ml of buffer [dilution 1 : 11]. This increases the range of measurable concentrations to 17,160 μg/ml.

In fact, one can prepare any dilution required partly by adjusting the volume of the buffer, partly by using an adequate loop. When the volume of the diluent buffer is 1,0 ml or less, the dilutions are prepared in MANDULA-type glass or plexiglass plates.

As regards the choose of the degree of dilution, it has to be noted that the above described soy bean medium requires a dilution of at least 1:20. At dilutions lower than 1 : 20 the ingredients suspended in the medium adhere to the surface of the disc, interfereing thereby with the diffusion of the antibiotic. If dilutions lower than 1 : 20 are used, the dilute sample should be centrifuged and a sample from the supernatant is transferred to the disc.

Parallel tests were made with solutions diluted by means of pipettes and with solutions prepared with a loop on the same agar plate. Comparison of the results revealed that the loop method was often more precise. The technique required is easy, only care should be taken that, when sampling, the loop should not be withdrawn from the fluid too rapidly, because then it will not take up the total volume required. Also, the loop should not touch the wall of the flask or container, since this, too, may decrease the volume of the sample.

The advantages of the method are seen in the following. The volume of the sample required for single determinations is very small (1 drop), the procedure can be carried out rapidly (within 1 minute), including sampling, diluting and placing of 2 to 4 parallels on a plate ; and, finally, the method is economical, since it does not involve the use of pipettes.

The method is now being adapted for aureomycin determination.

Summary

1. By combining the paper disc method with TAKÁTSY's spiral loop technique it has become possible to determine antibiotic concentration in a single drop of fermentation fluid. In this way great numbers of samples can be examined rapidly.

2. The method is well suited for selecting high antibiotic producer strains.

Acknowledgement

We are indebted to Msr. G. PÉNZES, of the Pharmacoindustrial Research Institute, for making available the test organism and for valuable information pertaining to this work.

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БЫСТРОЕ ОПРЕДЕЛЕНИЕ АНТИБИОТИКА ИЗ ОДНОЙ КАПЛИ ФЕРМЕНТАЦИОННОЙ ЖИДКОСТИ

И. ЛАСЛО и Г. САБО

Резюме

1. Путем объединения методов бумажного диска и разведения спиральной петлей можно определить антибиотик из одной капли ферментационной жидкости и таким образом открывается возможность быстрого исследования большого количества проб.

2. Метод пригоден для выбора штаммов, обладающих высокой способностью производства антибиотиков.

IMMUNIZATION EXPERIMENTS WITH DIFFERENT TYPES OF MUMPS VACCINE

By

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Since the basic observations of LEVENS and ENDERS [1] concerning adaptation of mumps virus to the developing chick embryo, considerable work has been done in order to produce a potent protective vaccine against mumps. During a period of about ten years several types of vaccine were developed, differing mainly in their virus content and in the method of inactivation [2, 3, 4, 5, 6]. Some experimental data concerning the immunizing properties of live attenuated virus are also available [7, 8]. At present the best results are obtained with the vaccine of HENLE et al. [7], inactivated by ultraviolet light and concentrated either by methanol precipitation or by Sharples centrifugation. These authors were able to protect about half of the vaccinated children with a single dose of 4 ml administered subcutaneously some weeks before the start of an epidemic.

All these experiments strongly indicate that the extent and duration of immune response highly depends on the amount of antigen inoculated. It seemed therefore interesting to examine whether it were possible to enhance the antigenic activity of inactivated mumps vaccines by some method other then increasing either the volume or the concentration of the antigen. Since adsorption of influenza vaccines to aluminium hydroxide gel has proved very satisfactory for that purpose [9], it was thought that it might be useful to perform similar experiments with mumps vaccines produced from the extra-embryonic fluids of infected hen eggs.

Materials and methods

Virus: The Enders strain of mumps virus adapted to the allantoic membrane of the developing chick embryo was used. The strain has been maintained in our laboratory by serial allantoic passages since about two years. During the study it was repeatedly identified by means of the haemagglutination inhibition test, using a standard hamster hyperimmune serum. The strain of virus and the homologous hamster immune serum were kindly supplied by DR. GY. TAKÁTSY (State Institute of Public Health, Budapest) to whom we are greatly indebted.

Mass production of virus for vaccination purposes was performed as follows. Eggs incubated for 8 days at 38° C were inoculated with 0,1 ml of a 1 : 100 dilution of fresh passage material (haemagglutination — HA — titre not below 1 : 128), simultaneously into the amniotic and allantoic cavities. After six days of further incubation at 35° C the eggs were chilled and the extraembryonic fluids harvested. Care must be taken to avoid bleeding because of the poor

elution of the virus from the red blood cells. The fluids were tested for sterility and haemagglutinating capacity, and those proving to be sterile and yielding HA titre not less then 1 : 128 were pooled. These pools were stored at 4° C until used, but never longer than ten days. During this period the appearence of a cloudy precipitate containing mostly urates was often observed. It was easily removed by low speed centrifugation, without any decrease in the HA titre.

The HA and haemagglutination inhibition (HI) tests were all performed following TAKÁTSY's micro method [10], with a 1 per cent suspension of washed chicken red blood cells. For purposes of the HI test, 4 haemagglutinating units of virus and sera inactivated at 56° C for 30 minutes were used. In all the tests a 2 plus reaction was taken as the end point.

Immunization experiments were performed in white Leghorn chicken each of which had at the start of the experiment weighed about 1000 g. The animals had not been vaccinated against Newcastle disease and following their arrival to our laboratory they had been quarantined for two weeks. During this time the serum of every animal was examined for the eventual presence of antibodies inhibiting HA by mumps or Newcastle disease virus. Only animals entirely lacking any of these antibodies were used in the experiments. Vaccination was always carried out by inoculation into the pectoral muscles.

Aluminium hydroxide gel was prepared from sodium hydrocarbonate and potassium aluminium sulphate of analytical purity.

All the bleedings referred to in the text were performed by cardiac puncture.

Experimental

Adsorption of the virus to aluminium hydroxide gel, in statu nascendi

According to the suggestion of ENDERS et al. [11], for adsorption of mumps virus from extracts of the parotid glands of infected monkeys, our adsorption experiments were also carried out using sodium hydrocarbonate and potassium aluminium sulphate as a gel producing system. Unfortunately in ENDERS' quoted paper no guidance had been given as how to prepare the gel and as to the quantitative relations of adsorption.

As a preliminary, a series of experiments was carried out in order to examine the influence of sodium hydrocarbonate and potassium aluminium sulphate on the virus and the chicken red blood cells, respectively. It was observed that sodium hydrocarbonate in a concentration of N/20 did not effect either the HA activity of the virus or the agglutinability of the red blood cells. Potassium aluminium sulphate, even at extremely high dilutions, caused the red blood cells to agglutinate and therefore its eventual effect on the HA activity of the virus could not be tested. It seemed therefore necessary to prepare the aluminium hydroxide gel under carefully controlled quantitative conditions to avoid the possibility of remaining free potassium aluminium sulphate in solution.

According to these findings the experiment was carried out as follows; a series of twofold dilutions of N/20 sodium hydrocarbonate solution was prepared. Two ml from every dilution were mixed with infected extraembryonic fluid of varying quantities ranging from 0,5 ml to 4,0 ml. After a thorough mixing the gel was prepared by adding 0,2 ml of potassium aluminium sulphate solution ten times the normality of the corresponding sodium hydrocarbonate solution used. The formation of the precipitate immediately occurred. After

ten minutes standing at room temperature the precipitate was centrifuged. The extent of adsorption was determined by titrating the supernatant. The dilution caused by the addition of sodium hydrocarbonate was, naturally, taken into account. A control consisting of the same reagents without virus was made parallel with the adsorption experiment. These latter supernatants proved always ineffective in agglutinating the red blood cells and they did not influence the HA activity of the virus. A typical adsorption experiment is presented in Table I. From the results the conclusion was drawn that under the experimental conditions employed the rate of adsorption seems to depend only on the quantitative relations between virus and gel.

Table I

Adsorption of mumps virus to $Al(OH)_3$ *gel*

Total $Al(OH)_3$ formed (mg)	Total amount of virus present (haemagglutinating units)				
	5,120.1	5,120.2	5,120.4	5,120.6	5,120.8
0,236/1	*96,1	96,1	93,8	81,7	62,5
0,236/2	96,1	95,4	81,7	62,5	50,0
0,236/4	96,1	81,4	62,5	50,0	50,0
0,236/6	84,4	62,5	50,0	50,0	50,0

* Per cent of virus adsorbed.

Similar experiments were carried out with virus preparations precipitated by methanol according to COX, as adapted to mumps virus by FORSTER and CASSON [12]. The results, presented in Table II., show that the rate of adsorption is much better if such a preparation is used, probably because of the smaller quantity of the adsorbable impurities present.

Table II

Adsorption of mumps virus purified by methanol precipitation to $Al(OH)_3$ *gel*

Total $Al(OH)_3$ formed (mg)	0,472	0,472	0,472	0,472	0,472
Total amount of virus present (haemaggl. unit)	20,400	20,400.2	20,400.4	20,400.8	20,400.16
Per cent of virus adsorbed	100	100	99,61	96,90	93,80

These experiments have shown that, if conditions are carefully chosen, a considerable quantity of virus may be adsorbed on very little gel. Considering that, according to the Hungarian state standards, the maximum gel content of an adsorbed vaccine must not exceed 8 mg/ml, our method permits the production of a concentrated gel-adsorbed mumps virus vaccine.

Immunogenic effect of the gel adsorbed mumps vaccine. In order to evaluate the immunogenic effect of the gel adsorbed mumps vaccine, comparative experiments were carried out using an unadsorbed vaccine as control.

Preparation of the gel adsorbed vaccine. As a starting material virus pools as described above were used. To two volumes of N/50 sodium hydrocarbonate one volume of extraembryonic fluid was added. Under constant stirring, 0,2 volumes of N/5 potassium aluminium sulphate were slowly given to the mixture.

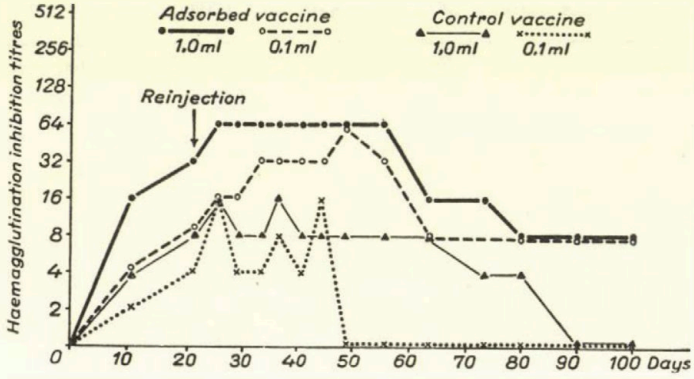

Fig. 1. Immunogenicity of different mumps vaccines. (Series I.)

The precipitate was collected by centrifugation and resuspended in buffered saline (pH 7,4) of 1/10 of the original virus volume, by homogenization in a Waring blendor for 3 minutes at 6000 RPM. To the homogenisate formaldehyde was added in a final concentration of 0,4 per cent. The extent of adsorption was about 95 to 96 per cent as calculated from the HA titre of the supernatant and the original virus fluid, respectively. The vaccine thus prepared was tested for sterility and safety in mice and guinea pigs.

The control vaccine was also prepared from the virus pools described above. The material was purified by centrifugation and the virus was inactivated by addition of formaldehyde in a final concentration of 0,4 per cent. After sterility and safety tests the vaccine was ready for use without any further treatment.

Immunization experiments were carried out in two different series.

In the first series, four groups of ten animals each were used. Two groups were inoculated with 1,0 and 0,1 ml, respectively, of the gel-adsorbed vaccine. The other two groups were injected with the control vaccine in a similar way and served as controls. Reinjection with the same dose as the first one was made

on the 21st day of the experiment. Blood was taken on every fourth day after the first injection ; the immune response was recorded by titration of the HI antibody content of the sera.

In the second series the experiments were performed as in the first series, with the only difference of administering the second dose sixty days after the first one.

Results and discussion

The results obtained in the first series are summarized in Fig. 1. The curves show the mean values of the HI titres of the four experimental groups,

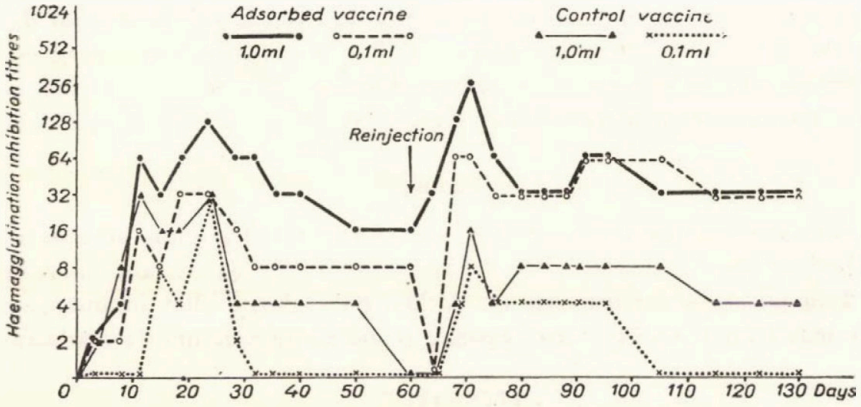

Fig. 2. Immunogenicity of different mumps vaccines. (Series II)

as plotted against time. The results show that the highest degree of antibody production and persistence has been initiated by the 1 ml doses of the ten times concentrated gel-adsorbed vaccine. No difference could be detected between the immunogenic activity of 0,1 ml of the gel-adsorbed and of 1 ml of the control vaccine during the 21 days following the first injection. After administration of the second dose, 0,1 ml of the gel-adsorbed vaccine has proved far more effective than 1,0 ml of the control, though both vaccines contained the same amount of mumps antigen. 0,1 ml doses of the control vaccine have produced only a moderate and short term immune response.

The results of the second experimental series are presented in Fig. 2. The immunogenic activity of the different vaccines permits of the following conclusions to be drawn. 1,0 ml of the gel-adsorbed vaccine had induced a satisfactory antibody formation attaining its peak at the 21st day following vaccination. The antibody titre then slowly decreased in about forty days to 1 : 16. On the sixtieth day the injection was repeated. As a result, a marked increase occurred in the HI antibody titre of the animals. In a few days this had fallen to a level of about 1 : 32 to 1 : 64 and persisted at that value until the end of

the experiment. Between the immune response induced by the first injection of 0,1 ml of gel-adsorbed and 1,0 ml of control vaccine, there was no marked difference, though the gel-adsorbed antigen has proved to be a little more effective. After the injection has been repeated on the sixtieth day a short negative phase was observed in the animals which had received 0,1 ml of the gel-adsorbed vaccine. This was followed by an increase in the antibody titre in both the gel-adsorbed and the control groups. After 0,1 ml of gel-adsorbed vaccine the immune response was of about the same order as after ten times the volume of the same vaccine, whilst in the group injected with 1 ml of control vaccine, only a lower and shorter antibody response has taken place. These results show that the immunogenic activity of inactivated mumps vaccine is considerably enhanced by adsorption to aluminium hydroxide gel, and the later the second dose is given the better the immune response. Similar observation concerning tetanus toxoid have recently been published by ERDŐS [13]. 0,1 ml of the control vaccine had a very moderate effect even in this type of experiment.

Summary

A method has been presented for the adsorption of mumps virus on aluminium hydroxide gel from the infected extraembryonic fluids of embryonated eggs.

Immunization experiments in chicken have shown that immunogenicity of the inactivated mumps virus vaccine is markedly enhanced by adsorption.

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ЭКСПЕРИМЕНТАЛЬНАЯ ИММУНИЗАЦИЯ ВАКЦИНАМИ СВИНК РАЗЛИЧНОГО ТИПА

Ш. КОХ

Резюме

В работе описывается метод адсорбции вируса свинки, культивированного в эмбриональных жидкостях зараженного яйца, к гелю гидроокиси алюминия. Автор путем иммунизации цыплят доказал, что эта адсорбция значительно повышает иммуногенное действие инактивированного вируса свинки.

THE USE OF SPIRAL LOOPS IN SEROLOGICAL AND VIROLOGICAL MICRO-METHODS

By

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The classical method of serological titration, that described by WIDAL, involves the use of pipettes and tubes for making serial dilutions. In virus research, where serological titration is a common procedure, the classical method is gradually losing ground in favour of techniques in which dilutions are made in grooves of plates made of glass or of plastic material. Such techniques have been described by NARTSISSOV [1], SALK [2], as well as by VAN DER VEEN and MULDER [3]. The advantage of the plate technique is that it facilitates supervision of parallel reactions and that plates are considerably easier to handle than tubes. The technique has been found particulary useful in virus research, since it is not negligible how much has to used from costly immune sera and antigens.

FULTON and DUMBELL [4] have developed a complement fixation test in which drops of reagents are brought together on a plain plastic plate. A drawback of the method is, however, that it cannot be used in other types of serological test and that serial dilutions must be prepared separately in tubes and transferred therefrom onto the plate.

The favourable experience obtained with my own serological micro method [5] over a period of years has induced me to present a detailed description of its technique.

The essence of the method is that serial dilutions are made in micro volumes, instead of by pipettes and tubes, by means of a calibrated spiral loop in the grooves of plastic plates. The diluent and the reagents are measured in by means of a calibrated instillator.

Equipment*

Spiral loop. This is made of heatresistant, elastic wire and is so formed that the volume of fluid taken up is determined almost exclusively by the space surrounded on every side by spiral wire. This is possible since the coiling is rather dense and thus the fluid taken up has no free surface of such a size which would possess a surface tension capable of influencing the

* All the equipment described may be purchased from Orvosi Műszer KV. Budapest.

volume of fluid. The loops (Fig. 1) take up 0,025, 0,05, 0,1 and 0,2 ml of fluid, respectively, with a maximum error of ±2 per cent. The loops are provided with a light metal holder.

Plates. These are made of translucent plastic, measure 8×68×130, or 8×95×130 mm in size and contain 6×12 or 8×12 grooves, 6 mm in diameter each. The grooves have a funnel shaped bottom and can take up 0,15 ml fluid.

Instillator. This is a small rost-free metal tube enclosed in plastic and its tip is so formed that the single drops of any fluid which has the same or nearly the same surface tension and specific gravity as those of water (physiological salt solutions, diluted solutions of proteins and, in general, aqueous solutions not containing an organic solvent), are 0,025 ml in volume. The instillator is provided with a cone by means of which it can be attached to the end of an adequately drawn-out ungraded pipette (Fig. 2).

The essence of the spiral loop dilution method. The diluent is measured in the grooves by means of the instillator. The single volumes are equal with, or 2, 3 *etc.* times that of the loop, depending on the steps of the dilution series to be prepared (1 : 2, 1 : 3, 1 : 4 *etc.*). With the flamed and cooled loop the surface of the fluid is touched, on which the fluid fills the space enclosed by the spiral. Surface tension will keep the fluid taken up in the loop. The loop filled with fluid is dipped into the first groove and rotated therein determinedly, causing the fluid introduced to be evenly mixed with the diluent. From groove 1 fluid diluted to the required level is transferred to groove 2 and the procedure is repeated until the desired number of dilutions have been made. In practice we usually work simultaneously with 6 loops.

Technique of dilution with the spiral loop

The loops are flamed with a Bunsen burner, partly to sterilize them and partly to remove fat, since a fat-contaminated loop does not take up fluid. The technique of flaming, however, is not identical with that used in bacteriological work. Care should be taken *not* to heat the loop to red heat. The following procedure has proved most suitable for this purpose. Six loops are flamed simultaneously, rotating the holders so as to heat each loop as evenly as possible. If any of the wires begins to glow, flaming is discontinued without delay. After flaming, the loops are allowed to cool. Meanhwile the diluent is measured in the grooves (Fig. 3), or, if there is someone to assist us, it is practicable to work with alternating series of loops. The cool loops are immersed into the diluent (Fig. 4) by which they become filled. The surface of the fluid must not be foamy lest bubbles enter the loop. The loop should not be immersed deep into the fluid ; it suffices to touch the fluid with the free tip of the loop, since capillary attraction fills it satisfactorily. Removal from the fluid should be carried out slowly or by touching the loop to the wall of the tube. If the loop is withdrawn rapidly a droplet of varying size will remain hanging on it, while an adequately withdrawn loop has no fluid on its outer surface. Sudden shaking or repeated touching of a wet surface by the loop will make it to release the fluid. The loops holding the diluent are introduced into the grooves constituting the first row of the plate, wherein they are rotated firmly for a short time, as described above

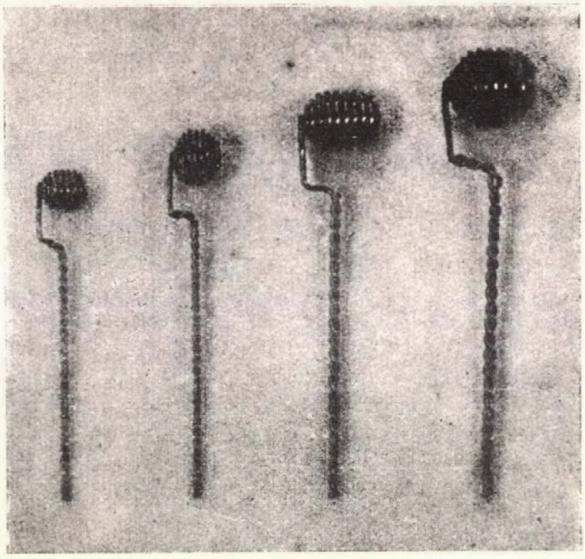

Fig. 1. Spiral loops, holding 0,025, 0,050, 0,1 and 0,2 ml

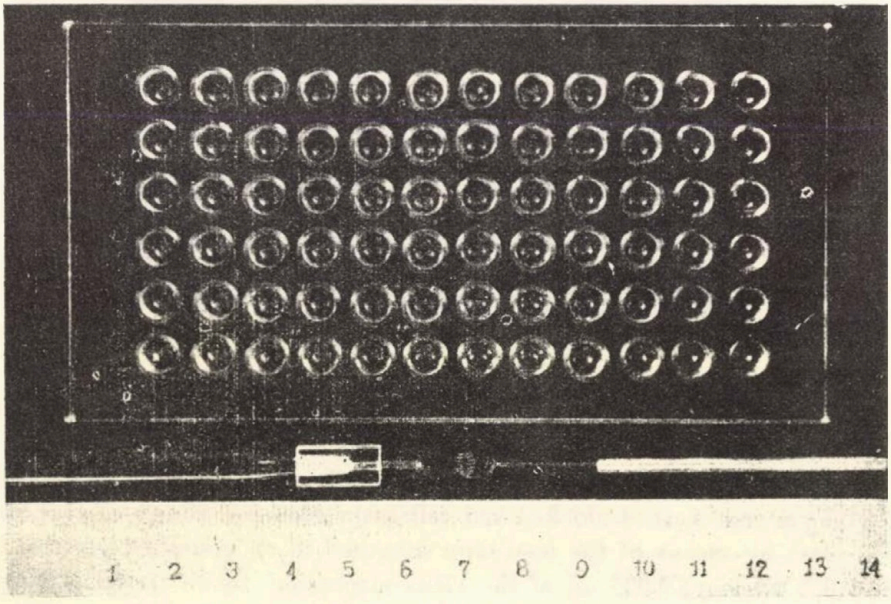

Fig. 2. Above: Plastic plate. On the left, instillator complete with pipette. On the right, 0,025 ml spiral loop

(Fig. 5). Then the loops are removed from row 1 to make the further dilutions (Fig. 6). Having completed the dilution series, the residual fluid is removed from the loops by means of tissue paper (Fig. 7) and the loops are flamed.

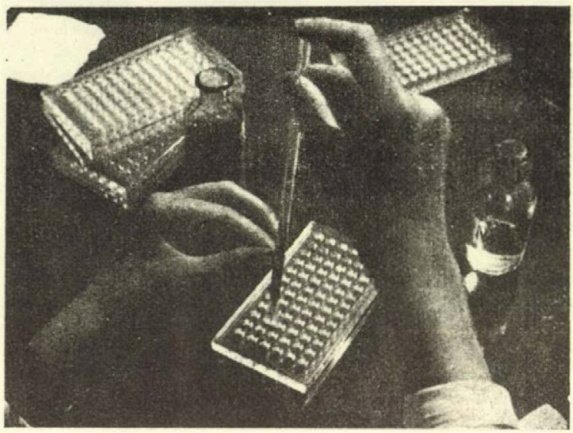

Fig. 3. Dripping in of diluent

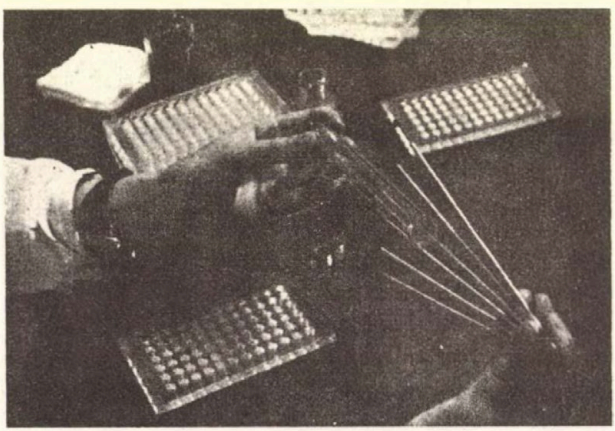

Fig. 4. Uptake of fluid with loop

The commonest titration procedures in virus research

Haemagglutination (HA) test. In informative titration, 0,05 ml (two drops) of a 0,5 per cent washed chicken red cell suspension are added to each groove of a plate by means of the instillator attached to an ungraded pipette. Using the 0,025 ml loop, 0,025 ml of the virus suspension to be tested is taken up and diluted serially in as many grooves as desirable. In this case threefold dilutions are made, and the dilution values are 1 : 3, 9, 27, 81, etc. If we want

to obtain a precise end point, another series is set up, diluting the first member of this 1 : 5, instead of 1 : 3 (by adding 0,1 ml = four drops to groove one). Thus, the two series will contain dilutions of 1 : 3, 5, 9, 15, 27, *etc.* The advantage of this parallel titration is that the end point is attained with a smaller number of intermediate dilutions, so that the error will be smaller and still

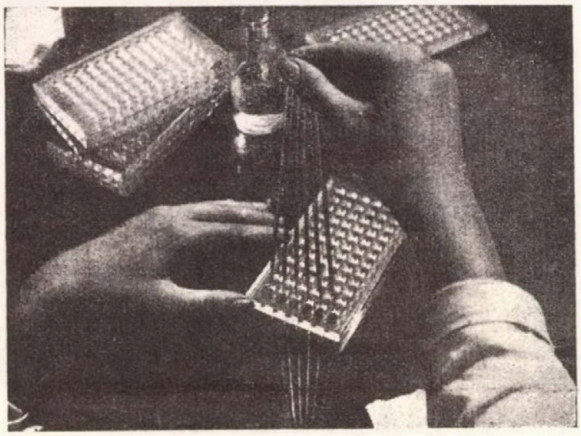

Fig. 5. Insertion of loops into grooves

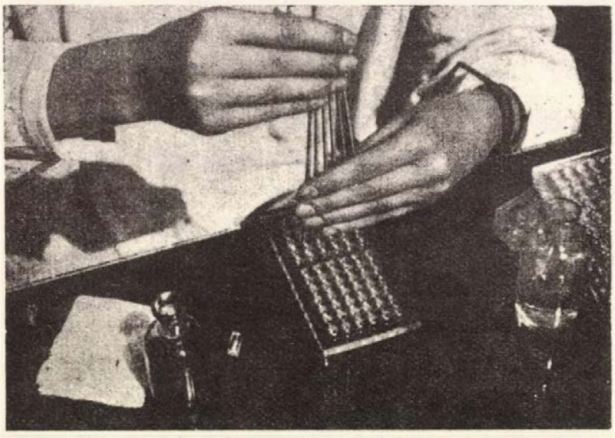

Fig. 6. Transfer of loops in making dilutions

the dilution series will be more protracted than with a twofold dilution series. After making the dilutions the plates are incubated at room temperature and when using chicken red cells the results are read after 30 to 35 minutes. The appearence of the reaction is shown in Fig. 8. In the presence of agglutination, an even coat of red cell network is seen at the bottom of the groove, as

compared with no, or partial, agglutination, where a dense disc of sharp outlines or a smaller disc with agglutination halo is formed. If partial haemagglutination designated with one to three crosses (+, ++, +++) is taken into consideration, an accurate estimate can be made of the HA titre of the suspension.

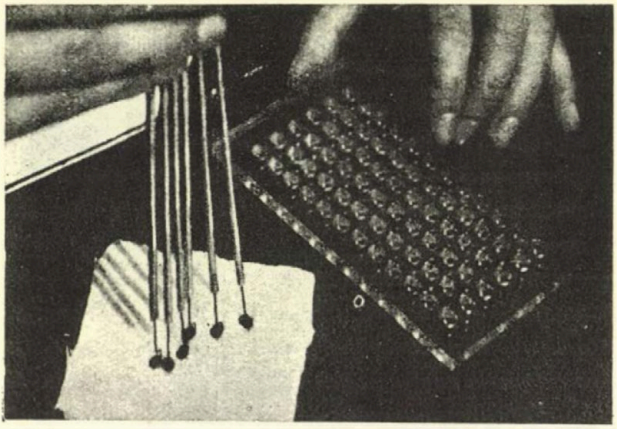

Fig. 7. Blotting of fluid from loops

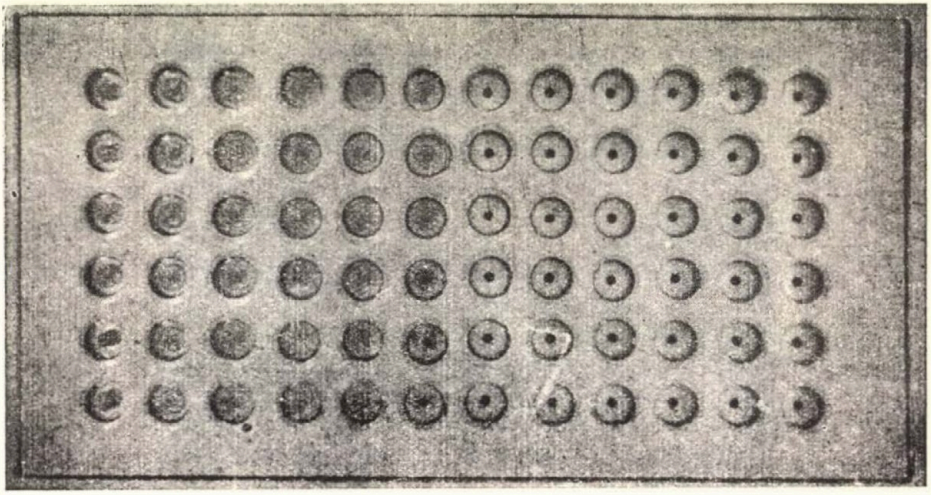

Fig. 8. Appearance of haemagglutination reaction after settling

A titration of this type is illustrated in Fig. 8, for 3 parallels each. It can be seen that agglutination is complete (++++) at dilution 1 : 729, it is negative at dilution 1 : 2187 (first 3 rows). In the series beginning with dilution 1 : 5 (rows 4 to 6), a one cross (+) agglutination is obtained for dilution 1:1215. If the

end titre is taken to be the 50 per cent agglutination (++), the calculation will be the following. The difference between the reciprocals of the dilutions causing partial (in the above example, +) and total (++++) agglutination is 1215 — 729 = 486. Dividing this by 3 (i. e. by the difference in the number of crosses) we obtain the one-cross value (162) Subtracting this from 1215, or adding its double to 729, will give the reciprocal of the dilution causing ++ agglutination, *i. e.* that of the end point, which in this case is 1053.

Incubation time may be considerably shortened by shaking the plates for one-half to one minute and by centrifugating them in a suitably arranged centrifuge (Fig. 9) at 500 r. p. m. for 10 secs. Then the plates are placed in the vertical position, when agglutinated red cells remain at the bottom of the grooves, while from negative grooves the red cell disc flows out. (Fig. 10). Grooves showing partial discharge mark the end point of HA titration.

For determining HA titre, the viral material is diluted directly in the red cell suspension proper. (See above.) This method has been chosen not only because of its simplicity, but also because the results obtained by diluting virus in physiological saline and by subsequent addition of red cells are not easily reproducible, especially when dilute or old viral suspensions are titrated. The error in titrating such suspensions appears to be due to adsorption of virus ; the rate of this depends on the quality of the material the loop is made of, or on the condition of the oxidized layer coating it. If virus is diluted in a red cell suspension, the great affinity of the virus to red cells will prevent its adsorption onto the wire. No such adsorption occurs when immune sera are diluted. In the HA test described, in the first members of the dilution series the red cell suspension becomes somewhat diluted by the virus material added. This dilution is negligible in the later members of the series. To avoid dilution of the red cell suspension, to the first grooves of each titration series we may substitute the drop of the 0,5 per cent red cell suspension by one drop of a 1 per cent suspension.

Haemagglutination inhibition (HI) test. Using the 0,025 ml loop, the serum to be tested is diluted in 0,025 ml (one drop) of physiological saline and to each groove are dripped 0,025 ml of a virus suspension containing 8 HA units. Serum and virus are mixed by repeated tapping of the rim of the plates (Fig. 11). After 15 minutes of incubation at room temperature, 0,025 ml of a 1 per cent red cell suspension are added and the plates are shaken again. After 20 to 30 minutes at room temperature the results are read. A two-cross (++) reaction is considered as the end point of HI titration.

The 8 HA units are titrated in the following way. The HA titre of the virus containing allantoic fluid is precisely determined in 3 parallel titrations as described above, except that after completing the dilution series 1 drop of physiological saline is added to each groove. By dividing the reciprocal of the titre by 8, the reciprocal of the dilution desired is obtained. This dilution is

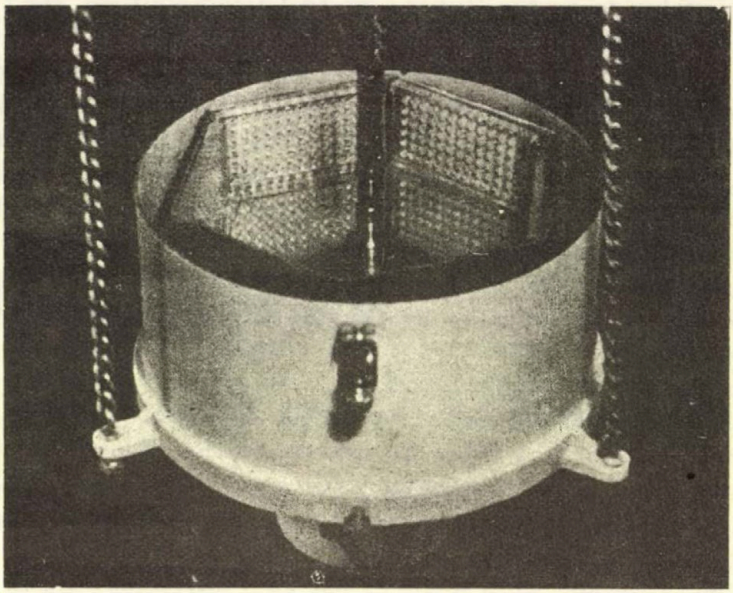

Fig. 9. Centrifuge for plates

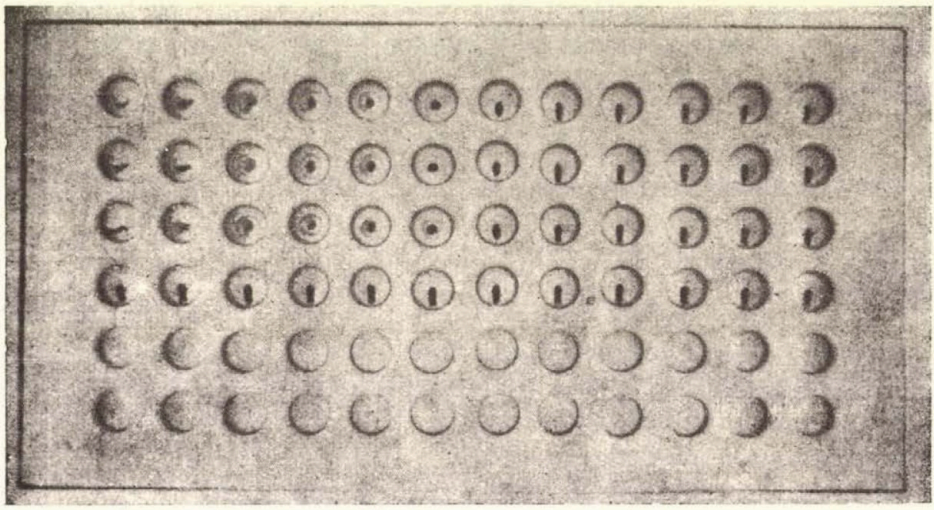

Fig. 10. Appearance of virus haemagglutination after centrifugation

titrated again in 3 parallels. The titration procedure consists of the following steps. Into the first grooves of the plate are measured 0,05 ml (2 drops) of a 1 per cent chicken red cell suspension, while to the other grooves a 0,5 per cent chicken red cell suspension is added. Using the 0,05 ml loop, a twofold dilution

series is made, then 1 drop of physiological saline is added to each groove. The dilution becomes suitable for inhibition when its titre is 1 : 8, *i. e.* when the third member of the dilution series gives a two-cross (++) haemagglutination. The titre of the viral suspension used for inhibition is checked at the time when the serum is being diluted, or, in prolonged experiments, at frequent intervals.

Complement fixation test. Using the 0,025 ml loop, a serial dilution is made of the test serum in 0,025 ml of physiological saline. After adding 1 drop of antigen of titrated concentration or a similar amount of complement, the plates

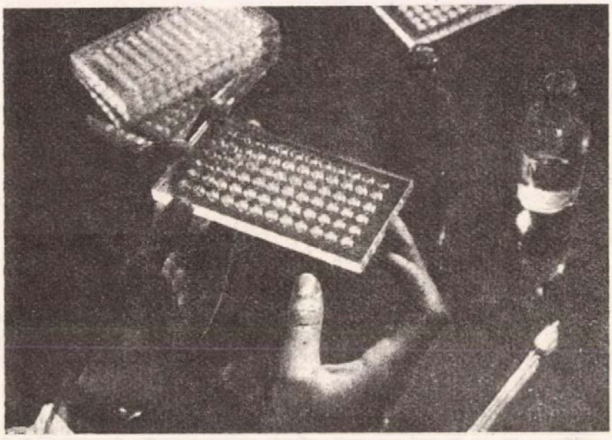

Fig. 11. Mixing of reagents

are incubated at 37° C for 20 minutes. After removal from the incubator 1 drop of the haemolytic system (containing equal amounts of a 5 per cent sheep red cell suspension and of titrated haemolysin) is added, and the plates, after shaking, are put back into the incubator for 20 minutes. Lysis, complete or partial, can be clearly visualized by placing the plate before a dark background (Fig. 12). The anticomplementary effect of serum is titrated in the same way, except that instead of antigen one drop of saline is added to each serum dilution. This quantitative titration will provide for an evaluation of specific fixation, despite a lower anticomplementary effect of the serum.

Complement titration is performed in two steps. Of complement diluted 1 : 20, doses increasing by 0,1 ml from 0,1 to 1,0 ml are added to 10 tubes, and the volume made up to 1 ml with physiological saline. To each of 10 grooves in row 1 are added 1 drop of negative serum diluted 1 : 2 and 1 drop of antigen used in the main reaction. To row 2 are added 1 drop of antigen and 1 drop of physiological saline ; to row 3, one drop of negative serum and 1 drop of saline ; and to row 4, two drops of physiological saline. At rectangles to each

row, 1 drop of each dilution of the complement is placed. After shaking and incubation at 37° C for 20 minutes, one drop of the haemolytic system is added. After another 20 minutes of incubation the result is read and the dilution of complement which has caused complete lysis in all four rows is used in the main experiment. If we want the main experiment to be less sensitive, more concentrated complement (by 10 to 20 per cent) should be used.

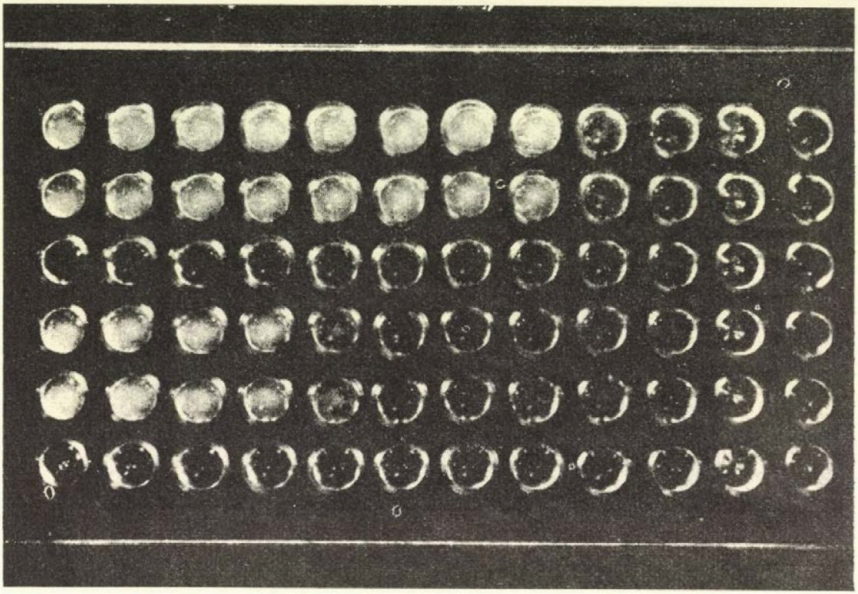

Fig. 12. Appearance of complement fixation reaction

The cold complement fixation test is carried out by the same technique, except that phase 1 is maintained at +4° C for 18 hours. The dilution of complement to be used in the reaction is determined by cold fixation.

The complement is preserved by an equal volume of a mixture of 12 per cent sodium acetate and 4 per cent boric acid and is stored in a refrigerator. It will maintain its activity for about 4 to 5 weeks, without any significan decrease in titre.

Sheep red cells are preserved with a modified ALSEVER solution [6].

The advantages of the loop method

First of all, it is much *more rapid* than WIDAL's dilution technique. Dilution is a completely mechanical procedure, not involving tiresome pipetting. Its technique is simple and with little training one can use six loops simulta-

neously, for diluting different materials or for making several parallels of one material. In general, the reactions can be read sooner and are easy to supervise. The plates occupy less space, can be cleansed rapidly and thus the method is especially advantageous in routine work. (One assistant can carry out 100 to 150 titrations within 1 hour). Moreover, the method is *exact*, for several reasons. (1) The volume of fluid measured varies within less wide limits of error than with measuring by pipettes; (2) error due to smearing of more concentrated solutions on the wall of pipette is absent, since the entire surface of the loop that had been in contact with the more concentrated solution is washed into the diluent. Finally, (3) diluting with the loop is perfectly devoid of subjective error of measurement. The method is *economical*, insofar as it does not involve the use of pipettes and an almost unlimited number of dilution series can be prepared with 12 loops, without being necessary to leave one's seat. There is no washing, preparation or sterilization of pipettes. The required volume of reagents is approximately one-tenth the usual.

The loop is superior to the pipette in every case when minute volumes of fluid have to be measured or diluted. Thus for example, very small volumes of blood taken from the finger tip or from laboratory animals can be taken up and diluted for quantitative work. Several samples of serum can be taken from centrifuged blood without causing thereby the serum to become turbid. The use of calibrated loops is advantageous in paper chromatography and electrophoresis, since minute volumes of fluid can be spread out precisely and evenly over the surface of the paper (SZALAY [7]).

In view of the above advantages, the loop technique is now in extensive use, especially for HA and complement fixation tests. The micro complement fixation test has been employed, for instance, by FARKAS and associates [8, 9, 10], by SZÖLLŐSY, IVÁNOVICS and HORVÁTH [11], by SZÖLLŐSY, ÁBRAHÁM and ALFÖLDI [12], as well as by FAZEKAS DE ST. GROTH [13] in influenza virus studies; by DÖMÖK *et al.* [14] in typhus, by TAKÁTSY and SZAFIR [15] in mumps, by DÖMÖK [16] in Coxsackie virus, by KÖRNYEI [17] in foot-and-mouth disease, and by ELEK and VIZY [19] in brucellosis studies. HORVÁTH and ALFÖLDI [18] have employed the spiral loop technique in their phage titration method, while LÁSZLÓ and SZABÓ [20] have used it in their rapid method for determining antibiotics in a single drop of fermentation fluid. The method has proved of particular value in our immune serum absorption experiments [21, 22], since only minimum amounts were required of costly virus preparations.

Summary

A new serological micro-method has been described, in which serial dilutions are made in grooved plastic plates by means of specially devised calibrated spiral loops, instead of with pipettes. There are loops capable of holding 0,025

to 0,2 ml of fluid. A calibrated instillator serves for measuring in diluent and reagents. A detailed description has been given of the commonest micro-methods used in virus research (HA, HI, complement fixation tests), as modified by the author.

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НОВЫЙ СЕРОЛОГИЧЕСКИЙ МИКРОМЕТОД

ДЬ. ТАКАЧИ

Резюме

Описываем новый серологический микрометод, по которому серийные разведения производим в углублениях пластмассовой пластинки, применяя вместо пипетки специальную, калибрированную, спиральную петлю. Петлей можно взять 0,025–0,2 мл жидкости. Замер разбавителя и реагентов производится с помощью калибрированного капельника. Таким способом разведения имеется возможность постановки наиболее часто применяемых в вирусологии реакций (реакции гемагглютинации, торможения гемагглютинации и связывания комплемента) с необходимой точностью и при этом достигается также большая экономия материалов и времени.

RECENT STUDIES OF THE ANTIGENIC STRUCTURE OF INFLUENZA VIRUS BY THE ANTIBODY ABSORPTION TEST

By

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In a previous report [1] we had described a simple technique for immune serum absorption tests and had discussed the quantitative relations of influenza virus-antibody union. Subsequently [2] the immune serum absorption test has been employed in investigations into the antigenic structure of influenza virus. Strain-specific sera have been prepared by repeated absorption and it has been found that the A-prime strains isolated recently in this country were inhibited by sera thought to be specific for earlier Hungarian strains, while they were considerably less inhibited by sera specific for foreign strains. From this the conclusion has been drown that the strains isolated recently in Hungary had originated mainly from older Hungarian strains. As to the antigenic structure of influenza virus, it has been concluded that its high variability is due not to a variation in part antigens already present, but rather to the appearence of new part antigens, associated with a gradual disappearence of older ones.

In the present report studies on Hungarian A-prime strains will be discussed. We endeavoured to find the answers to the following questions.

1. In what measure are new part antigens demonstrable in Hungarian strains and is the appearance of new part antigens related to a disappearence of older ones?

2. What is the sequence of events in this transformation and is it possible to demonstrate the variability of a strain within the course of an epidemic?

3. In what measure do strain-specific sera lend themselves for rapid antigen analysis?

Materials and methods

Strains of Virus. These were: Budapest (in the following): Bp. 4/49, Hung. 10/51, Bp. 7/51, Bp. 6/52, Hung. 15/53, Hung. 16/53, Hung. 17/53, Bp. 19/53, Bp. 20/53, Bp. 21/53, Bp. 22/53, Bp. 23/53, Bp. 24/53, Bp. 25/53, Bp. 26/53, Bp. 27/53, Bp. 28/53, Bp. 1/54, Bp. 2/54, Hung. 3/54, Hung. 4/54, Bp. 5/54, Hung. 6/54, Czech 1/54 and 2/54.

Method of isolation. Throat garglings obtained on the first or second day of ilness were treated with antibiotics and inoculated in 0,1 ml volumes into the amniotic sac of 8-day and 11-day chicken embryos. After incubating at 35° C for four days, the amniotic fluid was titrated against guinea pig, chicken and rat erythrocyte suspensions.

Erythrocytes. Citrated, four times washed chicken, guinea pig and rat erythrocytes were used, the latter two for isolation experiments only.

Immune sera. Hyperimmune sera were produced in chicken, 8 to 10 weeks old. The sera were prepared as follows. 1,0 ml of a purified virus preparation with a HA titre of 1 : 2000 to 1 : 5000 was intravenously injected twice in an interval of one week. A third inoculation followed after 8 weeks of rest and the animals were killed by bleeding twe weeks later. In this way we succeeded in producing sera of a higher titre than those obtainable with a larger number of inoculations at shorter intervals. The sera were stored at —6° C.

The *haemagglutination* (HA) and the *haemagglutination inhibition* (HI) tests were made by the spiral loop dilution method [3].

Virus preparations. The method for making concentrated and purified virus preparations has already been described [4, 5]. The only modification was that with strains carried over a smaller number of egg passages (whose egg titre was relatively low), red cell adsorption was carried out by using a lower red cell concentration and that elution was made in a volume of physiological saline four times that of the red cell sediment. In this way the viral titre of the eluate reached the degree where virus was precipitated when its suspension had been diluted with distilled water.

The technique of the *immune serum absorption test* and the method used for peparing *strain-specfic sera* have been described [1, 2]. In the present immune serum absorption experiments, attempts were again made to carry out absorption with an «optimum» quantity of virus, *i. e.* with a quantity capable of absorbing all heterologous antibody without leaving behind an excess of virus. Virus was diluted by means of the spiral loop, adding in every case 0,025 ml of virus to an equal volume of physiological saline. To this were then added 0,025 ml of serum with a HI titre of 1 : 500 to 1 : 1000. Sera with a higher titre were added to virus suitably diluted. The serum-virus mixture was repeatedly shaken during the 15 minutes of incubation at 37° C.

After adding two drops (0,05 ml) of physiological saline to it, the mixture was mixed again and centrifuged at 500 r. p. m. for 5 minutes in a special device constructed for holding the plexiglass plates [2], causing thereby the precipitate formed (the virus-antibody complex) to settle. In previous experiments [2] this titration had been considered to be a preliminary procedure, but in the present cross absorption tests it was the main experiment, since to test the absorbed serum against two strains no great volumes were required. Absorption could thus be carried out in a single step.

Experimental

First we examined Hungarian strains for the appearance of new part antigens and for the parallel disappearance of old ones.

Strains Bp 4/49, Hung 10/51, Hung 2/52, Hung 15/53 and Hung 3/54 were selected, they being prototypes of strains isolated in different years. Samples of antiserum to each of the test strains were absorbed separately with purified and concentrated virus prepared from each strain and it was examined in what measure the single absorbed sera were capable of reacting in HI tests with all of the strains mentioned.

Table I is a schematic presentation of the antigenic structure of the different strains, according to our working hypothesis. The assumed single parts of antigen and the antibodies to them are designated with Arabic numerals. The part antigens presumably present in strain 4/49, the one that had been isolated first, are designated with the numerals 1 to 6, while those of strains isolated later are indicated by the numerals 2 to 7, 3 to 8, etc., to emphasize that a new part antigen appears and the oldest one disappears. Thus, absorption of the antiserum to strains 4/49 with strain 3/54, for example, will result in the removal of antibodies to part antigens common to the two strains (in the diagram part antigens 5 and 6) and those antibodies will remain to which strain 3/54 contains

Table I

Hypothetical scheme of the antigenic structure of influenza A-prime strains

Strain	Antigenic structure									
Bp. 4/49	1	2	3	4	5	6				
Hung. 10/51		2	3	4	5	6	7			
Hung. 2/52			3	4	5	6	7	8		
Hung. 15/53				4	5	6	7	8	9	
Hung. 3/54					5	6	7	8	9	10

no part antigens. From this it follows that the absorbed serum will react with the other strains to the same degree as they contain part antigens 1 to 4. It was expected that the HI titre of the absorbed serum will be the lowest against strain 15/53 and will increase gradually with the strains isolated earlier, since these contain more and more of such part antigens which are capable of reacting with the serum.

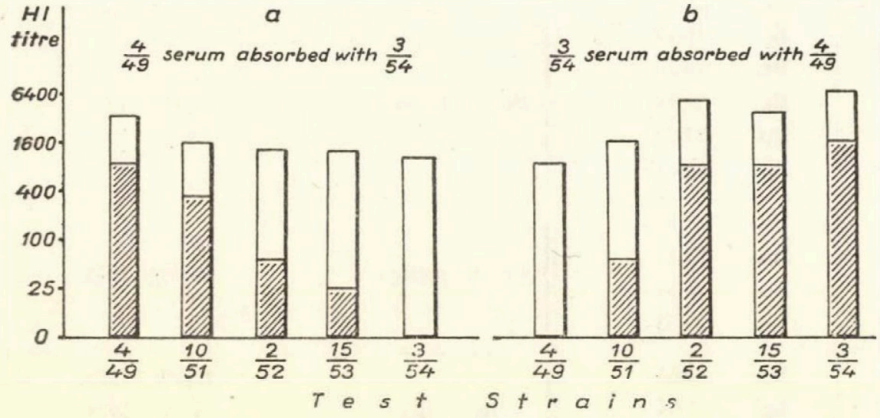

Fig. 1.

The validity of the above hypothesis has been examined experimentally. The results shown in Fig. 1/*a* agree well with our hypothesis. On the basis of a similar assumption, the above absorption test was carried out also inversely, *i. e.* the antiserum to strain 3/54 was absorbed with strain 4/49. The results agreed with what had been anticipated and the HI titres of the serum so absorbed have yielded a curve which was the mirror image of the one displayed in Fig. 1/*a* (Fig. 1/*b*), *i. e.* the titres of this serum were the

higher the more recently the strain had been isolated. The results of other absorption experiments essentially agreed with the hypothetical antigenic composition shown in Table I.

In further experiments the sequence of events in the alteration of antigenic structure demonstrated in the previous series was studied, and also whether it were possible to demonstrate changes in the antigenic structure of the pathogenic virus during one epidemic. The strains isolated from the 1953—54 influenza epidemic have proved to be suitable for this purpose. According to epidemiological data, the epidemic had broken out in West Hungary and, spreading East, it extended over the whole country within a few weeks. Preliminary typing showed that

Table II

Influenza virus strains isolated during the 1953—54 epidemic

No.	Designation of strains	Site of isolation	Date of isolation
1.	Hung. 14/53	Gyömöre (West)	12/11/ 1953.
2.	Hung. 15/53		
3.	Hung. 16/53		
4.	Hung. 17/53		
5.	Hung. 18/53		
6.	Bp. 19/53	Bp. A. college	12/12/ 1953.
7.	Bp. 20/53		
8.	Bp. 21/53		
9.	Bp. 22/53		
10.	Bp. 23/53		
11.	Bp. 24/53	Bp. B. college	12/19/ 1953.
12.	Bp. 25/53		
13.	Bp. 26/53		
14.	Bp. 27/53		
15.	Bp. 28/53	Bp. hospital	12/29/ 1953.
16.	Bp. 1/54		1 /5/ 1954.
17.	Bp. 2/54		1 /8/ 1954.
18.	Hung. 3/54	Miskolc (Northeast)	1 /8/ 1954.
19.	Hung. 4/54	Pécs (South)	1 /9/ 1954.
20.	Bp. 5/54	Bp.	1 /9/ 1954.
21.	Hung. 6/54	Debrecen (East)	1/15/ 1954.

Abbreviations: Hung. = Hungary, Bp. = Budapest

the pathogenic agent was A-prime virus. The data for the total of 21 strains isolated are shown in Table II.

The strains from the 3rd to 6th egg passages had been used for producing antisera by inoculation into chicken. They were then tested in HI cross tests to compare them with strains isolated earlier in this country. The results are shown in Table III. The difference between the single strains was expressed in terms of the «d» value, introduced by DÖMÖK et al. [6] The antigenic structure of two strains has been considered different only if the value of «d» was higher than 4. It can be read from Table III that strains isolated from the 1953—54 epidemic were more or less different from earlier Hungarian strains. They were the most distantly related to strains 4/49 and 10/51 and closest to strain 2/53. The new strains did not differ appreciably from each other in cross HI tests.

It had, however, been established in earlier studies that the immune serum absorption test may unmask differences in antigenic structure not shown by a cross HI test. In view of this fact the new strains have been subjected to cross antibody absorption tests. Concentrated and purified virus was prepared from each strain and absorption was carried out in five dilutions of virus by a micro method, as it has been described under «Methods».

The results are shown in Table IV The «d» values were calculated from the titres of sera obtained by cross absorption. Homologous absorption was also tested in every case and since its «d» values were on the average below ± 2, in cross absorption tests all values higher than 2 were considered significant. The data for cross absorption with strain 2/52 are also included in Table IV. The strain is obviously different from the new strains, as shown by the high «d» values, but there were appreciable differences also between the new strains. When single new strains were matched with all the other new ones, the average of the «d» values yielded was positive in every case. Relatively high values were given by strains isolated during the initial and final periods of the outbreak. It can be also read from Table IV that there were certain differences also between strains recovered at the same site and at the same time (strains 15/53 and 18/53, strains 19/53 and 23/53, as well as strains 24/53 and 28/53, respectively, had been isolated in the same place on the same day).

The purpose of the experiments with strain-specific sera was to determine the proportion of specific part antigens in old and new strains. This may namely permit to conclude as to the origin of the strains. Since it was assumed that a Hungarian strain had been responsible for the outbreak, the recently isolated strains were tested against strain-specific sera to earlier Hungarian strains.

The data in Table V were calculated as follows. $t = 4\ n_t + p$, where $t =$ = the numeral shcwn in the Table, $n_t =$ the number of the serum dilution showing complete (++++) inhibition in the titration series, and $p =$ the degree of inhibition in samples showing partial inhibition, expressed in crosses (+).

Table III

Comparison of A-prime strains isolated in 1953—54 with each other and with strains isolated earlier

(»d« values)

Strain	4/49	10/51	7/51	6/52	2/52	15/53	17/53	18/53	19/53	20/53	21/53	23/53	24/53	25/53	26/53	27/53	28/53	3/54	4/54	6/54
4/49		4	7	13	12	8	16	21	17	16	23	19	13	13	11	14	11	16	15	16
10/51			8	12	18	13	12	13	18	16	15	18	13	11	10	17	14	15	15	18
7/51				7	14	6	5	5	9	5	6	8	3	5	5	3	3	7	5	9
6/52					12	3	9	6	6	10	11	12	8	7	8	12	8	12	6	16
2/52						1	5	6	0	3	8	8	3	3	2	6	5	3	0	4
15/53							0	0	2	−1	4	3	2	1	0	3	1	−1	−2	1
17/53								1	3	2	4	3	3	−1	1	3	0	2	1	4
18/53									−1	0	0	2	−2	0	1	3	0	1	2	5
19/53										1	−1	2	−2	−1	1	4	0	0	1	3
20/53											1	3	0	−2	−1	0	0	2	1	5
21/53												2	1	0	2	4	1	0	2	5
23/53													1	1	2	2	0	0	3	4
24/53														−1	1	2	0	0	1	3
25/53															0	0	0	1	0	2
26/53																3	0	1	0	4
27/53																	2	1	3	4
28/53																		0	0	1
3/54																			1	1
4/54																				3
6/54																				

The data in Table V reveal that specific part antigens of earlier strains were not demonstrable, those of later strains in traces only, and that only those of strain 2/52, isolated in 1952, were definitely demonstrable. The same applies to the two strains isolated in Czechoslovakia early in 1954.

Table IV

Comparison of A-prime strains isolated in 1953—54 in cross antibody absorption test (»d« values).

Strain	$^{2}/_{52}$	$^{15}/_{53}$	$^{17}/_{53}$	$^{18}/_{53}$	$^{19}/_{53}$	$^{20}/_{53}$	$^{21}/_{53}$	$^{23}/_{53}$	$^{24}/_{53}$	$^{25}/_{53}$	$^{26}/_{53}$	$^{28}/_{53}$	$^{3}/_{54}$*	$^{4}/_{54}$	$^{6}/_{54}$	»d« sum	»d« Mean
$^{2}/_{52}$	—	21	12	>24	22	12	23	>24	18	14	16	>24	16	20	24		
$^{15}/_{53}$		1	4	9	9	4	5	—	3	8	6	7	8	3	9	75	6,2
$^{17}/_{53}$			0	7	3	1	8	2	0	2	2	8	4	2	11	54	4,2
$^{18}/_{53}$				0	0	5	1	3	0	6	12	0	8	14	12	77	5,9
$^{19}/_{53}$					2	3	2	4	2	5	1	2	6	8	6	51	3,9
$^{20}/_{53}$						0	6	3	0	4	3	0	4	2	1	36	2,8
$^{21}/_{52}$							0	3	0	4	0	2	1	9	8	49	3,8
$^{23}/_{53}$								−1	2	0	0	4	8	9	10	48	4,0
$^{24}/_{53}$									0	2	3	0	4	10	3	29	2,2
$^{25}/_{53}$										0	6	0	5	8	5	55	4,2
$^{26}/_{53}$											0	0	4	6	5	48	3,7
$^{28}/_{53}$												0	—	16	11	50	4,2
$^{3}/_{54}$*													−2	16	4	>72	>6,0
$^{4}/_{54}$														0	16	119	9,2
$^{6}/_{54}$															0	101	7,8

* $^{3}/_{54}$ serum was not available. Each value is the difference of the homologons and the heterologus »t value« (see p. 207) of the opposite serum.

Discussion

We have succeded in obtaining new experimental data to confirm our earlier view on the variability of the antigenic structure of influenza virus. According to this theory, new strains arise not by virtue of part antigen variation, but in consequence of a continuous, incessant transformation of their antigenic structure. In support of this view are the investigations into the behaviour of influenza A-prime virus in antibody absorption tests described by HIRST [7], as well as the facts that classical A strains are not any more involved in recent epidemics and that even an informative antigenic analysis infrequently fails to unmask differences between recently isolated strains and those recovered a few years earlier.

Table V

Influenza A-prime strains tested against strain-specific sera

Designation of strains	Strain-specifis sera				
	$^{4}/_{49}$	$^{10}/_{51}$	$^{7}/_{51}$	$^{6}/_{52}$	$^{2}/_{52}$
Bp. 4/49.	24	—	—	—	—
Hung. 10/51.	—	24	—	—	—
Bp. 7/51.	—	—	16	—	—
Bp. 6/52.	—	—	—	24	—
Hung. 2/52.	—	—	—	—	24
Hung. 15/53.	—	—	—	4	17
Hung. 16/53.	—	—	2	—	16
Hung. 17/53.	—	—	2	4	16
Hung. 18/53.	—	—	—	2	15
Bp. 19/53.	—	—	—	3	18
Bp. 20/53.	—	—	—	5	20
Bp. 21/53.	—	—	4	—	13
Bp. 22/53.	—	—	2	5	16
Bp. 23/53.	—	—	—	—	13
Bp. 24/53.	—	—	—	—	12
Bp. 25/53.	—	—	—	4	12
Bp. 26/53.	—	2	—	4	16
Bp. 27/53.	—	2	—	—	16
Bp. 28/53.	—	—	—	—	12
Hung. 3/54.	—	—	—	—	16
Hung. 6/54.	—	—	—	4	20
Hung. 6/54.	—	—	—	—	16
Czech. 1/54.	—	—	—	—	13
Czech. 2/54.	—	—	—	—	13

The sequence of events in this continuous change of antigenic composition is conceived as involving a continuous uptake of new, and simultaneous loss of old, part antigens by the strains. This is what we had endeavoured to illustrate in Table I and what has been borne out in the experiments (Fig. 1).

In connection with the tests against strain-specific sera it must be discussed whether such sera are worthy of that adjective. According to Table I strain-specific sera can be produced only against the strains which take place at the beginning or at the end of a row containing all the strains that had undergone every single phase of transformation because only they contain such old or new part antigens which are absent from other strains. Technical difficulties, however, have prevented us from isolating strains from every phase of transformation, although it were of little practical significance to relate specificity to

strains in too close relationship to each other. Thus, in practice we select strains distinctly differing in antibody absorption test and specific sera are so prepared that they should react only with the strains homologous with the selected ones. In case of new strains, it may be anticipated that they will be inhibited by the immune serum to that homologous strain from which they had developed. According to the above, our 1953—54 strains have to be derived from strain 2/52, because they contain that part antigen of this latter which is absent from other strains, including those isolated in 1952. This part antigen had been new in 1952, as indicated also by the fact that in 1953 the antibody inhibiting strain 2/52 was the one detected the least frequently among the population (FARKAS and HAMAR [8]). It is therefore understandable why the descendants of just that strain were capable of causing the next epidemic. The experiments have disclosed also the fact that the 1953—54 strains already differed from strain 2/52, in that they possessed a new part antigen. The two strains isolated in Czechoslovakia in 1954 also contained the part antigen common to strain 2/52. In view of the fact that in that country the epidemic broke out approximately one month after the onset of the epidemic in Hungary, it is highly probable that the strain causing disease in Czechoslovakia had developed from the Hungarian one.

If specific properties of the virus and specific immunity of the host are considered to interact in modifying the antigenic structure [9], it must be assumed that the virus will suffer some changes also in the course of a single outbreak. We have been unable to demonstrate such differences by HI tests; this appears to indicate that one single strain was essentially responsible for the epidemic. In support of this view is the fact that the epidemic spread at a constant rate all over the country, and circumscribed foci, such as had occurred *e. g.* in 1951, were not detectable [10, 11]. It therefore appears justified to state that the differences in cross absorption tests between the 1953—54 strains are attributable to changes which a single strain of virus had suffered during the epidemic.

The three strains recovered in the final stage of the epidemic were markedly different from other strains. This is only natural, since the former were isolated some two weeks later than had been the strains immediately preceding them in numerical order and, in addition, they were isolated in remote areas of the country, invaded by the epidemic at a later period. It is noteworthy that strain 4/54, isolated in Southern Hungary, was different from strains 3/54 and 6/54 too, although these were recovered almost at the same time in Northeast and East Hungary, respectively. It is thought that in its spread toward the South and East, respectively, the pathogenic strain had passed populations with different immunity and that this may have accounted for the two types of change in the properties of the strain.

In the knowledge of this continuous transformation of influenza virus, the direction of further research is seen not so much in the development of

more sensitive methods of demonstrating differences in antigenic structure, but rather in studies of deeper, mainly biochemical, relations of the changes in antigenic structure. In this connection it may be pointed out that the influenza virus, known to be the most variable among viruses, is the one having a relatively high carbohydrate content. It will be the task of further research to elucidate an eventual correlation between the variability of the virus and the hapten-like effect or carbohydrates.

Summary

The antigenic composition of influenza A-prime strains recovered in Hungary has been analysed in antibody absorption tests. The results have confirmed our earlier theory, that the variability in antigenic structure is not due to a variation of part antigens already present in the virus, but to the appearance of new, and the gradual disappearance of old, part antigens.

Such a continuous transformation of virus has been demonstrated by means of cross antibody absorption tests in strains isolated during the 1953—54 epidemic.

In tests carried out with strain-specific sera it has been shown that the 1953—54 epidemic had been caused by a strain which can be considered to be a direct descendant of strain Hungary 2/52. The same strain was responsible also for the epidemic occurring in Czechoslovakia one month after the Hungarian outbreak.

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НОВЫЕ ИССЛЕДОВАНИЯ АНТИГЕННОЙ СТРУКТУРЫ ВИРУСА ГРИППА МЕТОДОМ ИСТОЩЕНИЯ ИММУННЫХ СЫВОРОТОК

ДЬ. ТАКАЧИ и М. ХАМАР

Резюме

Анализом антигенных структур венгерских гриппозных штаммов типа A', проведенным методом истощения иммунных сывороток, авторы подтвердили сложившееся у них ранее предположение об изменении антигенной структуры вируса гриппа. По этому предположению это изменение не является вариацией уже ранее существующих антигенных частиц, а состоит из появления новых частиц антигена и постепенного исчезания старых.

При исследовании штаммов, изолированных во время эпидемии 1953–54 годов методом перекрестного истощения, наблюдалось такое постепенное изменение штамма.

С помощью штаммспецифических сывороток установили, что эпидемия 1953–54 годов была вызвана таким штаммом, который может считаться последственником выделенного в 1952 году штамма 2/52. Тот же штамм вызвал чехословацкую эпидемию, приблизительно месяц спустя после венгерской.

PRODUCTION OF PLAQUES IN MONOLAYER TISSUE CULTURE BY AUJESZKY-DISEASE (PSEUDORABIES) VIRUS

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The fact that animal viruses exert a cytopathogenic action also in tissue cultures, has stimulated us to study viral colonies starting out from viral particles, analogous with bacteriophage plaques. DULBECCO was the first to succeed in culturing virus in the form of isolated plaques on chicken fibroblast plates. This author worked with equine encephalomyelitis virus in 1952 [1] and since then his thechnique has been adapted for use with other viruses. So far, vaccinia virus (NOYES, [2]) and poliomyelitis virus (YOUNGNER, [3], DULBECCO [4]) have been grown in the form of isolated colonies in monolayer tissue cultures.

The cytopathogenic action of Aujeszky-disease virus (in the following Ay-virus) has been studied at our Institute for years [5, 6, 7, 8]. This is what has induced us to attempt to culture this virus in the form of isolated colonies in chicken fibroblast plates.

Virus. Strain XXV., the one involved in earlier studies [5, 6, 7, 8], was used. Viral material cultured in embryonic chicken tissue suspension was employed [5], using the extracts of 72-hour, homogenized and centrifuged cultures.

Materials and methods

Preparation of chicken fibroblast monolayers. The technique was essentially identical with that described by NOYES . After removing head and limbs, 11-day old chicken embryos were thoroughly rinsed with Gey's solution and 5 of the torsos were pressed through a mesh screen in a 50-ml Janet syringe. The mesh screen was mounted on a metal frame fitted to the syringe. It contained 18 nickel-coated wires per cm so that embryonic tissue pressed through it became suitable for this type of work. The homogenized tissue was taken up in 10 ml of Gey's solution in a centrifuge tube and was centrifuged at 700 r. p. m. for 30 sec. in a horizontal centrifuge. The supernatant containing chiefly erythrocytes and cell detriment was discarded. 7 ml of Gey's solution were added to the sediment, which was then suspended by drawing up and blowing out by a 10 ml pipette, 15 to 20 times in rapid succession. This was followed by centrifugation with 700 r. p. m. after 15 seconds. Isolated cells and smaller cell aggregates remained in the supernatant, while rougher particles were settled out. The fluid was decanted and allowed to stand in a test tube for 20 minutes, to remove rough parts still present.

The fluid was subsequently drawn up into a Pasteur pipette and the cell count was determined in a haemocytometer. In later experiments direct counting was replaced by estimation of optical density. The suspensions so standardized were then diluted with nutrient fluid, to contain 10 to 12 million cells per ml.

The tissue cultures were made in Petri dishes, 5 cm in diameter. Into each dish were measured 0,4 ml of chicken (hen) plasma and 0,1 ml of chicken embryonic extract, which were spread out evenly by means of a glass rod. After the plasma had coagulated, 3 ml of cell sus-

pension were added and the Petri dishes were incubated at 37° C for 24 hours. During this time the cells had settled satisfactorily to form an uninterrupted fibroblast network. 24-hour cultures prepared in the described manner were used in the experiments.

The nutrient fluid for tissue cultures consisted of Gey's solution, 40 per cent ; horse serum, 40 per cent ; and chicken embryonic extract, 20 per cent. The horse serum used had been inactivated at 56° C for 30 minutes and its pH was adjusted to 7,4 by letting CO_2 pass through it. The nutrient fluid contained 100 units of penicillin and 100 μg of streptomycin per ml. The embryonic extract was diluted with equal parts of Gey's solution. The hen plasma contained heparin, 1 in 50 000.

Experimental

In order precisely to determine the number of infective particles, the culture fluid was carefully sucked off from 24-hour cultures and replaced by 0,5 ml of a suitable dilution of virus (10^{-4} to 10^{-6}). The cultures were allowed to stand in an incubator for various lengths of time to facilitate adsorption to cells of

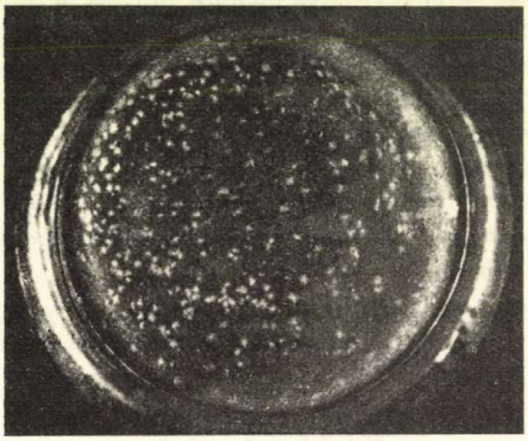

Fig. 1. Monolayer tissue culture infected with 10^{-4} dilution of viral material. About original size. Photographed in transillumination

virus particles. The virus-bearing fluid was then sucked off, 1 drop of heparinized chicken plasma was placed at each of four different sites in the culture, while 1 drop embroynic extract was dripped onto the centre. After mixing by gentle motion, the entire surface of the cultuie was coated with a thin layer of fibrin. To the infected and fibrin-coated cultures 3 ml of nutrient fluid were added and cultivation was continued for another 72 hours. Necrotic foci marking the site of virus particles appeared as pale spots. The foci can be made more conspicuous by vital staining of live cells. To this purpose the nutrient fluid is sucked off the culture and is replaced by 1 ml of Gey's solution containing neutral red, 1 in 5 000. Intact cells stain well within 1 or 2 hours, greatly facilitating the counting of plaques (Fig. 1.).

Adsorption times of virus are shown by the data in Table I.

Table I

Plaque counts for different adsorption times

Dilution of virus	Adsorption time hours	Plaque count for single plates	Average plaque count
10^{-4}	1	20 ; 30 ; 20 ; 26	25,25
	2	28 ; 20 ; 32 ; 26 ; 25	28,20
	3	30 ; 35 ; 32 ; 40 ; 36	34,60
	4	31 ; 29 ; 34 ; 38	33,00
	6	35 ; 39 ; 43 ; 30	36,75

Although the variations of the 2 to 6 hour results are within the limits of experimental error, they still indicate that the 1-hour adsorption time is insufficient. For this reason in later work 3-hour adsorption times were employed. An adsorption time of 3 hours' or longer duration is permissible with Ay-virus, since its multiplication does not begin until after a latency of about 16 hours [7].

Titration of Ay-virus containing material by the plaque-count technique has been carried out on 25 occasions. The relations are illustrated by the detailed results of 4 experiments, presented in Table II.

In the experiments shown in Table II. the infective particle count for the single viral materials was determined in 3 to 5 monolayer tissue cultures pre-

Table II

Infective particle counts for four different viral materials

Experiment No.	Dilution of virus	Plaque count on single plates	Mean plaque count	Deviation of single values		Particle count for 1 ml of vira material
				σ*	%	
1.	10^{-4}	75 ; 62 ; 50 ; 69 ; 65	64,2	$\pm$ 9,3	$\pm$ 14,4	$1{,}28 \cdot 10^6$
	10^{-5}	8 ; 17 ; 5 ; 9 ; 7	9,2	$\pm$ 4,6	$\pm$ 50,0	$1{,}84 \cdot 10^6$
2.	10^{-5}	32 ; 23 ; 27 ; 30	28,0	$\pm$ 3,9	$\pm$ 13,9	$5{,}6 \cdot 10^6$
	10^{-6}	3 ; 5 ; 4 ; 2 ; 3	3,4	$\pm$ 1,1	$\pm$ 32,3	$6{,}8 \cdot 10^6$
3.	10^{-4}	30 ; 35 ; 32 ; 45	35,5	$\pm$ 6,7	$\pm$ 18,8	$0{,}71 \cdot 10^6$
	10^{-5}	5 ; 4 ; 4 ; 3	3,8	$\pm$ 0,84	$\pm$ 22,0	$0{,}76 \cdot 10^6$
4.	10^{-5}	21 ; 26 ; 23	23,3	$\pm$ 2,5	$\pm$ 10,7	$4{,}7 \cdot 10^6$
	10^{-6}	2 ; 1 ; 3 ; 2	2,0	$\pm$ 1,41	$\pm$ 70,0	$4{,}0 \cdot 10^6$

* Calculated on the basis of formula $\sigma = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$

pared simultaneously and the average was calculated from the results. Although the small number of determinations does not allow for a reliable statistical analysis, we still present the standard deviation of single values used in calculating the means. If only a few plaques had appeared on the plates, the standard deviation was considerable, apparently due to the fact that the formula employed cannot be applied to such borderline cases, and Poisson's equation would better reflect the relations. If many plaques had developed on the single plates, *(e. g.* at the 10^{-4} dilution in experiment 1), the value obtained was low, owing to the confluence of plaques. For these reasons, dilutions of virus causing 10 to 40 plaques to be formed in each plate are the most suitable.

In comparative tests 5 different viral materials were examined for infectivity both by the plaque-count and by the so-called dilution technique. In these experiments the cytopathogenic titres were determined by a procedure described in detail elsewhere [8], by serial dilution in test tube tissue cultures of minced chicken embryo hearts. The suitable dilutions of viral material were contained in 2 ml volumes of the nutrient fluid of tissue cultures. Consequently, the results obtained by the two techniques are comparable only if the viral particle count per 1 ml is multiplied by 2. The values so obtained agree well in order of magnitude with the reciprocals of the cytopathogenicity titres determined by the dilution method.

Table III

Infectivity of five different viral materials, as determined by titration in tissue culture and by particle count

Experiment No.	Dilution method	Plaque-count method			
	Virus titre	Virus dilution	Plaque count	Mean plaque count	Particle—count/ml
1.	10^{-6}	10^{-5}	3; 2; 3	2,6	$0{,}53 \cdot 10^6$
2.	10^{-6}	10^{-5}	2; 3; 4	3,3	$0{,}66 \cdot 10^6$
3.	10^{-7}	10^{-5}	32; 27	31,0	$6{,}2 \cdot 10^6$
4.	10^{-6}	10^{-5}	4; 5; 8	5,6	$1{,}31 \cdot 10^6$
5.	10^{-6}	10^{-5}	7; 3; 2; 1	3,25	$0{,}65 \cdot 10^6$

Summary

Monolayer tissue cultures prepared from chicken embryonic tissue have proved suitable for estimating the number of Aujeszky-disease virus particles. Adequately diluted viral material kept in contact with the cell layer causes circumscribed necrotic foci to be formed. The infective particle count, as determined by this method, agrees well with the titre determined by serial dilutions in chicken embryonic tissue cultures.

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ОБРАЗОВАНИЕ БЛЯШЕК В ОДНОСЛОЙНЫХ ТКАНЕВЫХ КУЛЬТУРАХ ПОД ДЕЙСТВИЕМ ВИРУСА БОЛЕЗНИ АУЕССКОГО

И. БЕЛАДИ и Э. СЁЛЛЁШИ

Резюме

Однослойные тканевые культуры, происходящие из тканей куриного зародыша, оказались пригодными для исчисления частиц вируса болезни Ауесского. Вирусный материал, разведенный до надлежащего объема и содержимый в контакте с клеточным слоем, вызывает образование ограниченных очагов некроза. Число определенных таким образом инфекционных частиц согласуется с титром вирусов, установленным в тканевых культурах куриных зародышей, путем серийных разведений.

APPARAT ZUR VERBESSERUNG DER AGARSCHEIBENTESTMETHODE

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(Eingegangen am 16. Juni 1955)

RAPER und Mitarbeiter hatten zur biologischen Messung der Antibiotikumerzeugung und Untersuchung des Wirkungsspektrums die Agarscheibenmethode [1] eingeführt, die seither auch zur Messung der von Pilzen und Streptomyces erzeugten Antibiotika verwendet wurde (z. B. [2]). Sie besteht im wesentlichen darin, dass in Petri-Schalen der Wirkungsstoff der auf Agarplatten gezüchteten, Antibiotikum produzierenden Mikroorganismen in der Weise gemessen wird, dass mit einem Korkbohrer aus solchen Kulturen Scheiben herausgeschnitten und auf die die Testbakterien enthaltende Agarplatte übertragen werden. Nach einer gewissen Inkubationszeit bildet der auf die Agarplatte diffundierte Wirkstoff rings um die Agarscheibe einen Hemmungs- oder lysierenden Ring. Das Verfahren wurde dadurch erschwert, dass es mit Hilfe des Korkbohrers nur mit einer langwierigen und schwierigen Manipulation möglich war, die Agarscheibe auf die in der die Testbakterien enthaltenden Petri-Schale befindliche Agarplatte zu übertragen. Ferner blieb die Agarscheibe häufig im Korkbohrer haften und konnte von diesem nur beschädigt entfernt werden. Eine Schwierigkeit ergab sich auch daraus, dass die Agarscheibe auf die die Testorganismen enthaltende Agarplatte nur mit einem besonderen Hilfsmittel gehoben werden konnte..

Wegen dieser Schwierigkeiten fand diese Methode zur Messung des Wirkstoffes, obwohl sie insbesondere zur Bestimmung des Wirkungsspektrums der im Nähragar gut diffundierenden Antibiotika sehr geeignet schien, wenig Verbreitung. Daneben schien die Methode auch nützlich, um die Veränderungen zu untersuchen, die sich im antibiotischen Wirkungsspektrum einzelner Stämme der genetischen Veränderungen unterworfenen Arten ergeben. Mit Hilfe eines Handinstrumentes konnten wir diese Methode wirksamer gestalten, worüber nachfolgend berichtet werden soll.

Mit Hilfe des auf Abb. 1 sichtbaren Apparates schneiden wir auf der Agarplatte eine Scheibe aus, heben die Scheibe mit dem Apparat heraus und setzen sie auf die die Testorganismen enthaltende Agarplatte. Der Apparat besteht aus einem Kupferzylinder (A), der die Aufgabe hat, die Agarscheibe zu schneiden. Deshalb ist einerseits sein unteres Ende scharf geschliffen, während

anderseits dieses scharfe Ende mit 8 Einschnitten in der Achsenlänge (B) und einer aufmontierten Spirale (C) im Ruhezustand um 2 mm kleiner ist als der ursprüngliche Durchmesser des Zylinders. Das andere Ende des Zylinders hat einen ebenfalls aus Kupfer hergestellten Griff, auf dem der Druckhebel des in den Zylinder eingesetzten Kolbens ruht (D). Dieser im Zylinder befindliche Kolben ist in der Mitte durchbohrt, und in seinem Bohrloch befindet sich eine Achse, die zur Schnittfläche des Bohrers hin eine besondere, durch Druck bewegliche Scheibe hält. In gegensätzlicher Richtung ist der Druckknopf (E) zu sehen, mit dessen Hilfe die Scheibe auch über den Rand der Schnittfläche hinausgeschoben werden kann. Diese Achsenscheibe befindet sich auf Grund einer

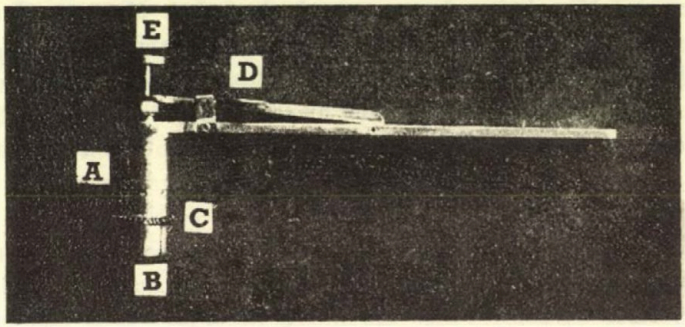

Abb. 1. Apparat zur Testierung mit Agarscheiben in Seitenansicht, in halber natürlicher Grösse

Spirale in Ruhelage in der Mitte des Schnittzylinders. Wird der Apparat in Tätigkeit gesetzt, drücken wir den Kolben herunter, wodurch sich die Rillen der Schnittfläche voneinander entfernen und der Durchmesser des schneidenden Teils um 2 mm grösser wird als in Ruhestellung. In dieser Lage schneiden wir die Agarscheibe heraus. Sodann bringen wir den Kolben in Ruhestellung, wodurch der Durchmesser der Schnittfläche um 2 mm kleiner und die Agarscheibe sozusagen umklammert wird. Nunmehr können wir die Agarscheibe ruhig herausheben und auf die Agarplatte übertragen, die sich in der den Testorganismus enthaltenden Petri-Schale befindet. Das Auflagen der Agarscheibe geschieht folgendermassen: Der obere Knopf wird so stark gedrückt, dass der Zylinder zusammen mit der Achsenscheibe über das Ende der Schnittfläche hinausreicht und die Agarcheibe abstösst. Mit einem einzigen Apparat können wir im Impfschrank Testierungen in Massen durchführen, wenn inzwischen mit 70%iger Alkohol desinfiziert wird. Im übrigen wird der Apparat vor Oxydation durch Vernickelung geschützt.

Mit Hilfe dieses Apparates können wir genaue vergleichende Messungen der Antibiotikumerzeugung vornehmen, wenn die Agarplatte für die des Antibiotikum produzierenden Mikroben folgendermassen hergestellt wird:

20 ml 2%iger Nähragar werden in Reagensgläsern sterilisiert und in heissem Zustand in eine normale Petri-Schale von gleichem Volumen gegossen. Nach Erstarren des Agars nehmen wir 5 ml Nähragar von gleicher Zusammensetzung und mischen 1 ml einer wässrigen Suspension des Antibiotikum erzeugenden Mikroorganismus oder seiner Sporen in der Weise dazu, dass die Temperatur des Agars 45° C sei. Die so bereitete Agarsuspension giessen wir auf die bereits hergestellte, 20 ml enthaltene Agarplatte, wobei wir darauf achten, dass sie sich auf der Oberfläche gleichmässig verteilt.

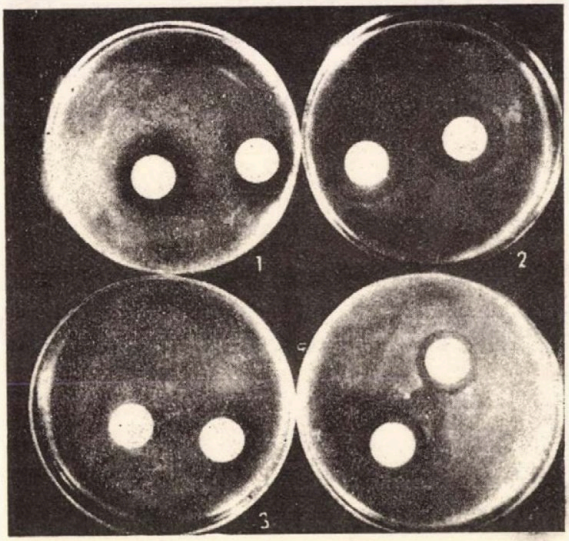

Abb. 2. 1 und 2: Messung der Antibiotikumerzeugung von zwei verschiedenen Streptomycesarten nach der kombinierten VINCENTschen Methode auf *B. subtilis*. 3 und 4: Messung derselben Arten nach der gleichen Methode auf *E. coli*

Auch den Erzeugungsprozess eines Antibiotikum produzierenden Mikroorganismus können wir folgendermassen feststellen. Die in der Petri-Schale befindliche Agarplattenkultur des zu untersuchenden Organismus wird bei der Temperatur gehalten, die den Bedürfnissen des antibiotikumerzeugenden Mikroorganismus entspricht. In bestimmten Zeitabständen schneiden wir Agarplatten heraus und testieren sie auf oben besprochene Weise. So gelangen wir zu der Produktionskurve des ein unbekanntes Antibiotikum erzeugenden Mikroorganismus. Nachdem wir den optimalen Zeitpunkt der Produktion während der Züchtung auf Agarplatte feststellten, schneiden wir zu dem Zeitpunkt, der dem Maximalwert der Antibiotikumerzeugung entspricht, dem Bedarf gemäss so viele Scheiben heraus, als wir zur Bestimmung des Wirkungsspektrums Agarplatten benutzen.

Bei sehr reichlicher Wirkstoffproduktion gehen wir in der Weise vor, dass wir auf den Boden der Petri-Schale Filtrierpapierscheiben von 18 mm Durchmesser legen, die etwas angefeuchtet werden, damit sie nicht von der Stelle rücken: auf diese legen wir die ausgeschnittenen Agarscheiben (s. oben), entfernen dann 6—8 Stunden später die Agarscheiben von den Filtrierpapierscheiben und legen das Filtrierpapier auf die Agarplatte in der den Testorganismus enthaltenden Petri-Schale, wie dies auf beigefügter Aufnahme zu sehen ist (Abb. 2). Wir vergleichen hier die Wirkstoffproduktion von zwei verschiedenen Streptomycesarten einerseits mit *B. subtilis* testierend (Bild 1 und 2), anderseits durch Messung auf *Escherichia coli* als Testorganismus (Bild 3 und 4). Es ist zu sehen, dass in bezug auf *B. subtilis* die Produktion in einem Falle grösser, im anderen Fall jedoch geringer ist, während auf *E. coli* gemessen das Antibiotikum der einen Art wirksam ist, das der anderen dagegen nicht.

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АППАРАТ ДЛЯ УСОВЕРШЕНСТВОВАНИЯ МЕТОДА АГАРОВЫХ ДИСКОВ

Я. ХОРВАТ

Резюме

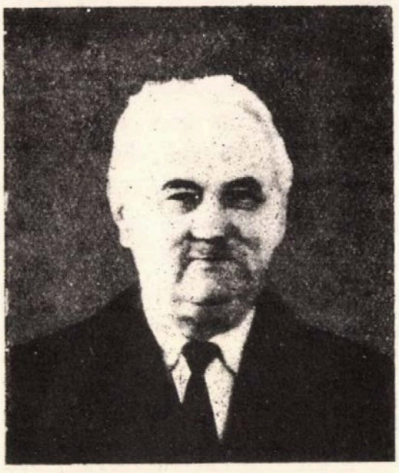
Метод агаровых дисков, введенный ранее для измерения действующего вещества и спектра действия антибиотических микроорганизмов, не получил распространения. Распространению препятствовало то обстоятельство, что при изготовлении дисков пробойниками для пробок оказывалось много недостатков (так, например, агаровый диск прилипал к пробойнику для пробок и повреждался при удалении его оттуда, в другом случае не удавалась выемка дисков и тд). С помощью ручного приспособления этот метод сделали более эффективным. Видимый на приложенном снимке аппарат состоит из медного цилиндра (А), служащего для вырезки дисков, ввиду чего конец его остро наточен. Посредством нарезок (В) и пружины (С), прикрепленной к боку цилиндра, режущую кромку можно урегулированно расширять и суживать на 2 мм. Расширение и суживание производится с помощью подвижного поршня, расположенного внутри цилиндра. Отдельный диск служит для выталкивания вырезанного агарового диска. На снимке видна его нажимная кнопка, обозначенная «Е».

Аппаратом работаем следующим образом: нажимаем поршень, вследствие чего нарезки режущей кромки отдаляются друг от друга и таким образом диаметр ее становится на 2 мм больше, чем в покойном положении. В этом положении вырезываем агаровый диск. Затем приводим поршень в покойное положение — когда диаметр режущей кромки уменьшается — и таким образом как бы захватываем ею агаровый диск и вынимаем. Таким способом переносим агаровый диск на поверхность пластинчатого агара, содержащего тесторганизм. Затем выталкиваем его с помощью нажимной кнопки «Е» и таким образом помещаем на агаровую пластинку.

Для обеспечения хорошей работы аппарата необходимо достаточно агаровой питательной среды и ее хорошее распределение. Для этого предлагаем следующее : в чашку Петри наливаем 20 мл 2%-ой агаровой питательной среды и после застывания накладываем на ее поверхность слой микроорганизма, производящего исследуемый антибиотик и взвешенного в 5 мл агаровой питательной среды. Методом агаровых дисков от времени до времени проверяем образование действующего вещества и таким способом можем составить и кривую производства, с помощью которой хорошо определяется спектр действующего вещества.

Если производство является очень высоким, то помогаем так, что вырезанные агаровые диски кладем на кружки из фильтровальной бумаги такого же диаметра и спустя 6—8 часов исследуем их надлежащим тесторганизмом на поверхности агара в чашке Петри. (Рис. 1 и 2.)

NACHRUF ÜBER DÁNIEL FEHÉR



Die Schriftleitung der Acta Microbiologica Hungarica teilt tief betroffen mit dass am 17. Februar dieses Jahres ein Mitglied des Redaktionskomitees, der zugleich Vizepräsident der Ungarischen Mikrobiologischen Gesellschaft war, Dr. Dániel Fehér, korrespondierendes Mitglied der Ungarischen Akademie der Wissenschaften, plötzlich verschieden ist.

Der Tod von Dániel Fehér bedeutet nicht nur für die Ungarische Mikrobiologische Gesellschaft und die Redaktion dieser Zeitschrift einen unersetzbaren Verlust, sondern ebenso für das ganze Gebiet der ungarischen biologischen Wissenschaft.

Dániel Fehér lebte 65 Jahre lang. Er wurde in Tekepuszta, im Komitat Győr geboren. 1912 erhielt er sein Forstingenieurdiplom auf der Hochschule für Forstingenieure in Selmecbánya. 1918 wurde er Assistent im Botanischen Institut derselben Hochschule. 1920 erwarb er sein Doktordiplom von Botanik und Chemie auf der Wiener Universität. Seine weitere Wirksamkeit entfaltete er im Botanischen Institut der Hochschule für Berg- und Forstingenieure in Sopron. So wurde er 1921 Adjunkt, sodann — als junger Mensch — 1923 ausserordentlicher öffentlicher Professor und schliesslich 1926 ordentlicher öffentlicher Professor. Er unternahm zahlreiche ausländische Studienreisen. 1934 unrd 1936 organisierte und führte er zusammen mit Kilian, dem Leiter der Biologischen Station in Algir, die französisch-ungarische Sahara-Expedition. Bis 1951 wirkte er als Hochschulprofessor, von da an bis zu seine plötzlichen Tod lebte er als Leiter des Bodenbiologischen Laboratoriums der Ungarischen Akademie der Wissenschaf-

ten nur für die wissenschaftliche Forschung. Ein Jahr vor seinem Tode wurde er zum korrespondierenden Mitglied der Ungarischen Akademie der Wissenschaften erwählt. Er war ein gründendes Mitglied der Ungarischen Mikrobiologischen Gesellschaft und nahm an der Verlagsarbeit der Gesellschaft bis zu seinem Tode teil.

Seine wissenschaftlichen Arbeiten erschienen 35 Jahre hindurch. Er schrieb 270 Abhandlungen, von welchen 130 in ungarischer und 140 in verschiedenen Kongressprachen herausgegeben wurden. Er schrieb 7 Bücher in ungarischer, eines in deutscher und eines in französischer Sprache. Die Mehrzahl der ungarischen Bücher waren Hochschullehrbücher aus dem Kreise der Botanik, Pflanzenbiologie, organischen Chemie und Bodenbiologie. Kurz vor seinem Tode erschien sein Werk «Talajbiológia» (Bodenbiologie), ein Handbuch von über 1200 Seiten, das hauptsächlich die neuesten Ergebnisse der Bodenmikrobiologie sehr ausführlich erörtert. In dieses Werk arbeitete er sein im Jahre 1933 bei Gustav Fischer in Berlin erschienenes und auch international bekanntes Buch «Mikrobiologie des Waldbodens» ebenfalls hinein. Letzteres fasste in seinem überwiegenden Teil die Resultate der Mikrofloraforschungen in verschiedenen Waldböden zusammen, welche Forschungen grösstenteils von Fehér und seinen Mitarbeitern durchgeführt wurden. Mit diesen beiden Büchern trug er viel zur Erschliessung der Problematik der Bodenmikrobiologie und zu den auf diesem Gebiet erzielten Resultaten bei. Ein besonderes Interesse erweckte das 1939 in Paris erschienene Buch «Microbiologie des sols sahariens», das die Ergebnisse der französisch-ungarischen bodenmikrobiologischen Sahara-Expedition veröffentlicht. Aus diesem Werk erfuhr die wissenschaftliche Welt, dass Mikroorganismen auch in vollkommen ariden Böden leben. Fehér gelang es, festzustellen, dass in der Sahara auf einer Stelle, wo es jahrelang kein Niederschlag gibt und die Vegetation fehlt, eine messbare Bodenatmung besteht, also auch dort eine Mikrobentätigkeit vor sich geht. Mit Mikroskop wies er die in diesen Böden tätigen Mikroorganismen durch die Cholodnysche Methode ebenfalls nach.

Der bedeutendste Teil seiner selbständig oder zusammen mit Mitarbeitern herausgegebenen wissenschaftlichen Publikationen erschliesst die Funktion der Mikroorganismen des Bodens. Er untersuchte die Gestaltung der Mikrobenzahl in verschiedenen Waldböden und später in landwirtschaftlich bestellten Böden. Im Zusammenhang damit deckte er den physikalischen und chemischen Zustand der geprüften Böden auf, um die Wechselwirkung zwischen diesen Faktoren und der Tätigkeit der Mikroben zu erkennen. Er stellte Zusammenhänge der Mikrobenzahl des Bodens mit der Bodenfeuchtigkeit und -temperatur fest (Fehérsches R-Gesetz). Er erforschte, in Einzelheiten gehend, die Fragen der Bodenatmung, Produktion organischer Stoffe, Stickstoffzirkulation, Phosphor- und Kaliumbewirtschaftung des Bodens, chemischen Reaktion (p_H) usw., usw. in der Arbeit der Mikroorganismen des Bodens.

Über die Mikrobiologie hinausgehend, führte er — dem Interesse eines echten Biologen entsprechend — auch auf anderen grossen Gebieten Forschungen durch. Von diesen sind der Gedanke und die bezüglichen Versuche erwähnenswert, laut welcher jedes Element Strahlen emittiert, die nur auf biophysikalischem Wege mit den tropistischen Krümmungen der Pflanzen messbar sind.

Dániel Fehér schrieb durch die tiefgreifende wissenschaftliche Tätigkeit seinen Namen für ewig ins Gedächtnis der ungarischen und internationalen bodenmikrobiologischen Wissenschaftlicher ein. Sowohl die Ungarische Mikrobiologische Gesellschaft als auch die Redaktion der Acta Microbiologica Hungarica werden seiner immer in Ehrfurcht gedenken. Wir können mit Überzeugung behaupten, dass sich die von ihm in Ungarn auf breiter Basis ausgeübte Bodenmikrobiologie durch seine ihm würdigen Nachfolger weiter entwickeln und das grosse Lebenswerk von Dániel Fehér nicht in Vergessenheit geraten wird.

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ÜBER DIE ZUSAMMENSETZUNG DES BEI DER PENICILLINPRODUKTION ENTSTEHENDEN MYZELS

Von

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Das für die Penicillinproduktion in grossen Ausmassen in Tiefkultur erzeugte Penicillium chrysogenium bildet auch eine beträchtliche Menge Myzel, das bei Beendigung der Fermentation von der Nährbodenflüssigkeit getrennt wird, so dass in den Betrieben als Nebenprodukt der Fabrikation täglich bedeutende Mengen Pilzkörper entstehen. Die Untersuchung dieses Nebenproduktes verspricht sowohl theoretisch wie praktisch interessante Resultate. Theoretisch ist seine Untersuchung deshalb interessant, weil wir zwar über die Natur der Gerüstsubstanzen der höher gearteten Pilze, ebenso wie über die der Hefen und Bakterien in der Literatur ziemlich viele Angaben finden und auch über die Körperstoffe der Aspergillus-Arten sowie gewisser Schimmelpilze einige Daten anzutreffen sind, sich jedoch mit der eingehenderen Untersuchung dieses Nebenproduktes der Penicillinproduktion bisher verhältnismässig wenige Autoren befasst haben. Seine praktische Bedeutung ist dadurch gegeben, dass einerseits das Myzel in einer auch vom wirtschaftlichen Gesichtspunkt bedeutenden Menge entsteht, anderseits die Substanz nach Literaturangaben und auch unseren eigenen Erfahrungen über wertvolle Eigenschaften verfügt.

Bekanntlich wird das Myzel auch in Ungarn schon seit einiger Zeit zur Herstellung von Ergosterin benutzt. Mit einer anderweitigen praktischen Verwendung des Stoffes hat sich einer von uns schon seit längerer Zeit beschäftigt. In der Mikrobiologischen Abteilung des Staatlichen Instituts für Gesundheitswesen sowie am Tierbestand des Instituts durchgeführte Versuche ergaben, dass der Myzelextrakt im Nährboden zahlreicher, tierisches Eiweiss beanspruchender pathogener Krankheitserreger das aus tierischen Eiweissstoffen hergestellte Pepton und auch Bouillon völlig zu ersetzen vermag. Es ergab sich ferner, dass die in der Mäuse- und Rattenzucht infolge mangelhafter Ernährung auftretenden Vermehrungsausfälle durch Verabreichung des Myzels verhindert werden können. Nachdem es sich ferner zeigte, dass das Myzel auch in grossen Mengen völlig unschädlich ist, benutzen wir es heute bereits zur Futterergänzung bei grösseren Tieren [3].

Das Myzel enthält laut Literaturangaben etwa 0,85% Ergosterin [1], ferner Biotin, Riboflavin, Nikotinsäure, Pyridoxin, Pantothensäure [2, 6],

Folsäure [6] sowie mehrere wichtige Aminosäuren, z. B. Histidin, Arginin, Lysin, Leucin, Isoleucin, Valin, Methionin, Treonin, Phenylalanin und Tryptophan [5]. Ausserdem wurde eiweiss-, stärke-, phosphorsäureester- und pektinabbauende Enzymwirkung nachgewiesen [7].

In der Literatur finden wir zahlreiche Angaben darüber, dass die Gerüstsubstanzen der Pilze aus Chitinstoffen bestehen. Frühere Autoren betrachteten diese Stoffe als identisch mit dem Chitin, welches das Gerüst der Insekten und Krebse bildet. So isolierte SCHOLL [8] aus dem Pilz Boletus edulis 5—6% «Chitin» mit einem, auf alkalischer Behandlung beruhenden Verfahren, das mit kleineren oder grösseren Modifikationen zur Isolierung der Gerüststoffe von Pilzen allgemein übernommen wurde. BROWN [9] stellte ebenfalls aus einer Citromyces-Art ein Gerüststoffpräparat her, das offenbar ausschliesslich aus N-Azetylglukosamin-Einheiten aufgebaut war.

Unter den sich mit der Untersuchung der Gerüststoffe von niederen Pilzen beschäftigenden anderen Autoren fand TAKATA [10], dass die Gerüstsubstanz des Aspergillus oryzae zu etwa $^1/_3$ aus »Chitin« besteht. MAY und WARD [11] sowie WARD und JANIESON [12] stellten aus Penicillium javaicum 25%, alkalischer Behandlung widerstehenden Gerüststoff her, dessen Stickstoffgehalt zwischen 4—5,4% variierte. Sie stellten fest, dass ein Teil des Stickstoffgehaltes der Gerüstsubstanz Bestandteil der N-Azetylglukosamin-Moleküle ist, sahen aber nicht als erwiesen an, dass der gesamte Stickstoff in dieser Form anwesend ist.

NORMANN und PETERSON [13] fanden in der 20% ausmachenden, gegenüber alkalischer Behandlung resistenten Gerüstsubstanz des Aspergillus fischeri 3,5% Stickstoff und schätzen den »Chitin«-Gehalt des Gerüststoffes auf 50%. Ihrer Ansicht nach besteht die laugenresistente Substanz der Zellwand aus einem Komplex von Hexosamin-, Glukose- und Azetylmolekülen bzw. -gruppen sowie aus lockerer gebundenen Glukosemolekülen.

Auch aus den Literaturangaben ist zu ersehen, dass die Gerüstsubstanzen der niederen Pilze von ziemlich komplizierter Zusammensetzung sind, und die Beurteilung dieser Angaben erfolgt nicht immer einheitlich. Da wir über die Gerüststoffe des bei der Penicillinproduktion entstehenden Myzels in der Literatur keine Angaben fanden, nahmen wir an, dass ihr Studium auf allgemeineres Interesse rechnen darf, da die ermittelten Angaben uns der Kenntnis der Gerüstsubstanzen der niederen Pilze näherbringen können.

Das nach Beendigung der Fermentation frisch ausgepresste Myzel ist eine schwach gelblichbraune Substanz von der Beschaffenheit eines hartgekneteten Teiges und hat einen charakteristischen, an Pilze erinnernden Geruch. An der Luft nimmt es erst eine grünliche Färbung an, dann wird es — vor allem an der Oberfläche — braun, wobei sich zum charakteristischen Pilzgeruch ein unangenehmer, an Buttersäure erinnernder gesellt. Das native Myzel enthält trotz seiner verhältnismässig konsistenten Beschaffenheit nur etwa 7% Trocken-

substanz. Nach vollständiger Austrocknung nimmt der unangenehme Geruch der Substanz ab, und der bezeichnende Pilzgeruch tritt wieder in den Vordergrund. Das Myzel bindet Wasser auffallend gut, so dass seine schonende Trocknung keine ganz leichte Aufgabe darstellt. Die ausgetrocknete Masse verliert teilweise ihre wasserbindende und Quellfähigkeit, wird aber nach Zusammenkneten mit der fünffachen Menge Wasser wieder von teigartiger Beschaffenheit.

Unsere orientierenden Versuche begannen wir mit dem im Fabrikbetrieb entstehenden Myzel. Die im Fabrikbetrieb auf übliche Weise entstehenden Myzelproben enthielten kein Eiweiss mehr, sondern nur amphotere, stickstoffhaltige Verbindungen, wahrscheinlich niedrigere Polypeptide oder eventuell Aminosäuren. Wir versuchten, Mannit oder Glykogen nachzuweisen, vermochten diese aber in der untersuchten Substanz nicht festzustellen. Löslichen und unmittelbar reduzierenden Zucker enthielt sie nur zu etwa 2,20%, doch fanden wir in ihr einen in Wasser löslichen, jedoch in Alkohol unlöslichen, 3,6% Stickstoff enthaltenden Stoff, der bei kräftiger Hydrolyse neben Huminstoffen reduzierenden Zucker bildete.

Nachdem das auf übliche Weise gewonnene Myzel an löslichen Kohlenhydraten so auffallend arm war und Eiweiss gar nicht enthielt, dürfte darin offenbar eine sehr starke Autolyse vor sich gegangen sein. Aus diesem Grunde entnahmen wir im Penicillin-Fabrikationsbetrieb unmittelbar nach der Auspressung aus der Presse eine neue Probe von 1 kg und legten diese unverzüglich in 3 Liter Azeton, um eine eventuelle Enzymwirkung zu verhindern. Nach 8 Tagen entfernten wir das Azeton, worauf der Rückstand je 2 Tage in 3 bzw. 2 Liter Äther gehalten, hiernach ausgepresst und der Rest im Vakuum bei 40° und dann über gebranntem Kalk getrocknet wurde.

Mit dieser Behandlung entfernten wir nicht nur das Wasser, sondern auch einen beträchtlichen Teil der Fettstoffe, so dass sich aus der verbliebenen, ziemlich leicht pulverisierbaren Masse mit Petroläther nur noch 1,52% Fett extrahieren liess. Mit der Analyse der nach Eindampfen der vereinigten azetonätherhaltigen Flüssigkeit zurückgebliebenen unangenehm riechenden teigartigen Masse haben wir uns nicht befasst. Der Aschegehalt der fettfreien Substanz betrug 11,9%, der Gesamtstickstoffgehalt 6,01%, der Kalziumgehalt 1,39%, der Magnesiumgehalt 0,86%, der Phosphorgehalt 2,35%, der Eisengehalt 0,38%.

Zur Untersuchung der Eiweissstoffe wurden Mengen von jeweils 1 g der fettfreien Substanz im Eisschrank nacheinander mit je 100 ml Wasser, 10%iger Kochsalzlösung und 0,2%iger Natriumhydroxydlösung extrahiert und der Stickstoffgehalt der gewonnenen Extrakte sowie des ungelösten Rückstandes bestimmt. Es ergaben sich folgende Resultate: Wässriger Extrakt 1,44%, salziger Extrakt 0,15%, alkalischer Extrakt 0,78%, ungelöster Rückstand 3,58%; zurückgewonnen insgesamt 5,95%; Stickstoffverlust im Laufe der Manipulationen 0,06%. Die Extrakte ergaben mit Trichloressigsäure und Sulfosalizylsäure schwache, aber entschieden positive Reaktion, doch stand die

Stärke der Reaktion anscheinend nicht im Verhältnis zum Stickstoffgehalt. Im wässrigen Extrakt kann ausser nicht-eiweissartigen Stoffen und Zersetzungsprodukten die Anwesenheit von Albuminen angenommen werden, während der salzige Extrakt über die Globuline, der alkalische Extrakt über die Azidalbumine Auskunft hätte geben müssen.

Aus vorstehenden Angaben können wir die Schlussfolgerung ziehen, dass auch von den löslichen Stickstoffsubstanzen des »desenzymatisierten« und schonend getrockneten Myzels höchstens die Hälfte als Bestandteil grösserer Moleküleinheiten anwesend sein kann, während die andere Hälfte, sofern sie eiweissartig ist, bereits in der letzten Fermentationsphase oder im Laufe der Auspressung Autolyse erleidet.

Zur weiteren Untersuchung des Verhaltens der Eiweissstoffe, ferner um für die Untersuchung der Gerüstsubstanzen, die weiter unten beschrieben wird, entsprechende Ausgangsstoffe zu gewinnen, nahmen wir mit den Myzelien auch einige Verdauungsversuche vor, wobei wir im grossen ganzen nach der bereits von VASTAGH und RÓZSA [4] angewandten Methode vorgingen.

Mengen von 50 g des entfetteten Myzels wurden in einen 2 Liter-Erlenmeyerkolben eingemessen, 0,5 g mit Wasser zerriebenes Pepsin von »2000-facher« Verdauungsfähigkeit dazugespült, mit Wasser auf etwa 1 Liter ergänzt, hiernach das pH mit 10%iger Salzsäure auf 4 eingestellt, das Gemisch mit 5 ml Toluol übergossen und mit einem Kochbecher bedeckt im Thermostaten bei 40° bis zum nächsten Tage stehen gelassen. Am folgenden Tage wurde die Flüssigkeit durch ein Filtertuch filtriert, der Rückstand schwach ausgepresst, sodann in den Kolben zurückgewaschen, das pH erneut eingestellt und die Verdauung mit Pepsin wiederholt.

Nach der Verdauung des ersten Tages war die fiiltrierte Flüssigkeit stark trübe, völlig undurchsichtig und braun, am zweiten Tage war das Filtrat fast durchsichtig und bouillonfarbig.

Nach der zweiten Pepsinverdauung wurde der Rückstand erneut in den Kolben zurückgewaschen, 1,5 g Kochsalz und 2 g mit Wasser zerriebenes Pankreatin zugegeben, mit Wasser auf 1 Liter ergänzt, das pH [mit Natronlauge] auf 9 eingestellt, hiernach auf die Oberfläche der Flüssigkeit 5 ml Toluol geschichtet und der zugedeckte Kolben bis zum nächsten Tage in den Thermostaten gestellt.

Nachdem die Verdauung in dieser Weise beendet war, wurde die Suspension durch ein Filtertuch filtriert, der Rückstand ausgepresst, zweimal mit je 500 ml Wasser gewaschen und das Waschwasser mit dem ursprünglichen Filtrat vereinigt. Wir bestimmten den Trockensubstanz- und Stickstoffgehalt sowohl der Verdauungssäfte als auch des unverdaulichen Rückstandes, wobei wir die Anwesenheit von Pepsin, Pankreatin und NaCl berücksichtigten. Den Stickstoffgehalt des Verdauungsrückstandes stellten wir mit 2,26% fest; dieser enthielt ausserdem 0,57% Phosphor.

Mit drei verschiedenen Substanzen nahmen wir vier Verdauungsversuche vor. Indem wir deren Resultate mit denen der unter Anwendung von kaltem Wasser, Kochsalz und Lauge durchgeführten Extraktionsversuchen verglichen, vermochten wir einige schätzungsartige Schlüsse zu ziehen. Aus der Tatsache z. B., dass bei der Verdauung etwa 80% des ursprünglichen Stickstoffgehaltes in Lösung gingen, während sich ohne Verdauung lediglich etwa die Hälfte extrahieren liess, können wir folgern, dass ungefähr $^1/_3$ des Myzels aus einer — sich in dieser Hinsicht eiweissartig verhaltenden — Substanz besteht, die durch einfache Extraktion nicht in Lösung überführt, aber mit Pepsin bzw. Pankreatin verdaut werden kann. Die direkt (ohne Verdauung) lösliche Substanz kann teils aus sich auch unter diesen Umständen lösendem Eiweiss, teils aus den Autolyseprodukten von Proteinen und anderen stickstoffhaltigen Stoffen, u. a. stickstoffhaltigen Oligosacchariden, bestehen (wir erwähnten schon, dass die Menge der letzteren mit 6—7% angenommen werden kann, ihr Stickstoffgehalt von 3,6% kann demnach 2—3% des Gesamtstickstoffs repräsentieren). Endlich nimmt ungefähr ein fünftel des Stickstoffes am Aufbau der Gerüstsubstanz teil.

Auf Grund dieser Feststellungen schien die Herstellung der Eiweissverbindungen des Myzels in reiner Form oder in grösserer Menge ziemlich aussichtslos. Infolgedessen haben wir uns mit dieser Frage vorläufig nicht weiter beschäftigt, sondern gingen zur Untersuchung der Gerüstsubstanz des Myzels über. Wir nahmen an, dass es sich um eine chitinartige Verbindung handelt. Ausser den bereits erwähnten Autoren haben sich mit Chitin auch noch zahlreiche andere befasst (15—25). Das im engen Sinne verstandene (d. h. von Tieren stammende) Chitin wird, mit Säuren gekocht oder durch Einwirkung der Enzyme einzelner Schneckenarten, zu N-Azetylglukosamin-Molekülen hydrolysiert, während es, mit konzentrierten Alkalien längere Zeit gekocht oder mit festem Kaliumhydroxyd bei 170—180° geschmolzen, desazetyliert wird, wobei jedoch die auf diese Weise entstandene und mit Chitosan bezeichnete Substanz ihre dem ursprünglichen Chitin ähnliche Struktur behält. Auch die Gerüstsubstanz der niederen Pilze soll eine dem Chitins ähnliche Verbindung sein.

Wir versuchten, die Gerüstsubstanz so zu isolieren, wie dies SCHOLL beim Boletus edulis vornahm. Die von diesem Autor beschriebene, im wesentlichen aus mehrmaliger Erwärmung mit 10%iger Kalilauge, danach mit Kaliumpermanganat bestehende Behandlung ergab jedoch sowohl in der Ausbeute (4,30—13,00%), als auch im Stickstoffgehalt (2,88—4,14%) derart abweichende Resultate, dass mit dieser Methode keine einheitliche, definierte, reproduzierbare «Chitin»-Substanz zu gewinnen war. Doch wurde unzweifelhaft klar, dass wenn wir unter Chitin einen Stoff verstehen, dessen Molekül allein aus N-Azetylglukosamin-Bruchstücken besteht, die Gerüstsubstanz des Myzels nicht als ein im engeren Sinne verstandenes Chitin angesehen werden kann, sondern dass — soweit die Analogie der Literaturangaben auf unseren Stoff bezogen werden

kann — angenommen werden muss, dass am Aufbau der Gerüstsubstanz ausser N-Azetylglukosamin-Molekülen auch stickstofffreie Einheiten teilnehmen. Ferner sehen wir, dass die mit erschöpfender Alkalibehandlung hergestellte Substanz nicht als natürliche Gerüstsubstanz des Myzels zu betrachten, sondern lediglich als künstliches Produkt der energischen Laugenwirkung aufzufassen ist, in dem das an Stickstoff verhältnismässig reichere Produkt dadurch zustande kam, dass die gegenüber der Alkalibehandlung weniger widerstandsfähigen stickstofffreien (Kohlenhydrat-) Stoffe abgebaut und herausgelöst wurden. Im übrigen ist dies für aus Hefe in ähnlicher Weise durch Alkalibehandlung gewonnene Polysaccharide schon von SEVAG und Mitarbeitern [14] angenommen worden.

Um zu entscheiden, ob das gewonnene Produkt zumindest auf Grund seines Azetylgehaltes als eine chitinartige Verbindung angesehen werden kann, musste auch der Azetylgehalt bestimmt werden. Zu diesem Zweck musste zuerst die Azetylgruppe vom Aminozucker abgespalten werden, wozu die Kalischmelze der Gerüstsubstanz am geeignetsten erschien. Am Schmelzpunkt des Kaliumhydroxyds ist nämlich eine Zersetzung des Azetyls nicht zu erwarten, während sich bei dieser Temperatur sowohl die niedrigeren wie die höheren Fettsäuren wahrscheinlich zersetzen. In Modellversuchen überzeugten wir uns davon, dass es sich tatsächlich so verhält.

Da sich die in der Literatur zur Bestimmung der Essigsäure angegebenen, als spezifisch bezeichneten Methoden (26, 27, 28, 29, 30, 31) für uns als ungeeignet erwiesen, waren wir genötigt, ein für unsere Zwecke geeignetes Verfahren auszuarbeiten.

In einen Nickeltiegel wurden 0,20—0,30 g Gerüstsubstanz eingemessen, mit 1—2 ml 0,5 n propylalkoholischer Kalilauge und etwa 0,5 g Kaliumhydroxyd am Wasserbad und hiernach im Trockenschrank fest eingetrocknet (wenn Propylalkohol zurückbleibt, entzündet er sich beim Schmelzen und verursacht Essigsäureverlust). Auf den Trockenrückstand wurden noch etwa 3 g pulverisiertes Kaliumhydroxyd gestreut, der Rückstand hiernach geschmolzen und 10 Minuten lang — bei möglichst niedriger Temperatur — in geschmolzenem Zustand gehalten. Die abgekühlte Masse wurde in wenig Wasser gelöst, in den Kolben des SCHULEK-VASTAGHschen Destillationsapparates gewaschen, 1 g Magnesiumsulfat zugegeben, in Anwesenheit von einigen Tropfen Methylrot mit 50%iger Schwefelsäure angesäuert und das Volumen stetig kleinhaltend etwa 60 ml Kondensat abdestilliert. Zum Destillat gaben wir einige Tropfen Phenolphthalein-Lösung und titrierten es mit 0,1 n Natronlauge bis zum Verbrauch von 9 ml. Hiernach wurde das Gemisch aufgekocht, 1 Minute lang (zur Vertreibung der Kohlensäure) schwach kochend gehalten und dann die Titrierung kalt beendet. In den Modellversuchen gewannen wir 98—101% der eingemessenen Essigsäure zurück.

Bei der Untersuchung der mit Laugenbehandlung gewonnenen Gerüstsubstanzen nimmt jedoch der festgestellte Essigsäuregehalt mit der Verringerung

des Stickstoffgehaltes zu und weicht vom berechneten Wert immer mehr ab. Die nächstliegende Erklärung hierfür dürfte sein, dass beim Schmelzen mit Kalilauge ausser Essigsäure und Kohlensäure auch andere sich mit Wasserdampf verflüchtigende Säuren entstehen, die sich unter den verhältnismässig schonenden Bedingungen und der kurzen Dauer der Schmelze nicht zersetzen.

Es ist bekannt, dass Essigsäure der Oxydation durch Kaliumpermanganat sowohl im sauren als auch im alkalischen Milieu weitgehend widersteht, während die anderen mit Wasserdampf flüchtigen organichen Säuren unter ähnlichen Umständen bis zu Kohlensäure oxydiert werden. Von dieser Tatsache überzeugten wir uns auch experimentell durch Zugabe von 0,1 n Essigsäure. In Kenntnis dieser Tatsache modifizierten bzw. ergänzten wir unsere Methode folgendermassen :

Nach Beendigung der beschriebenen Schmelzung und Destillation wird das Destillat in den Kolben des SCHULEK-VASTAGHschen Apparates zurückgewaschen, 3 ml 25%ige Kalilauge und 1—2 g pulverisiertes Kaliumpermanganat zugegeben, der Kühler aufgesetzt und die Hälfte der Lösung in etwa 30 Minuten abdestilliert (Glasperlen!). Der Auffangkolben wird ausgetauscht, zum Inhalt des Destillierkolbens 3 ml 50%ige Schwefelsäure sowie 2 g Magnesiumsulfat gegeben, und indem wir das Volumen auf einem Niveau von 10—15 ml halten (und die abdestillierte Flüssigkeit in kleinen Teilmengen ersetzen) 60—70 ml Destillat abdestilliert (wobei darauf zu achten ist, dass sich der Inhalt des Destillierkolbens nicht allzu sehr einengt, weil aus dem Kaliumpermanganat Manganheptoxyd entweicht und in das Destillat Permangansäure gelangt). Die Titrierung des Destillats wird in der schon beschriebenen Weise in Anwesenheit von Phenolphthalein vorgenommen. Wenn wir die Essigsäuremenge nicht einmal annähernd kennen, muss das Auskochen der Kohlensäure vor Beginn der Titrierung durchgeführt werden ; nachdem jedoch hierbei ein gewisser Essigsäureverlust möglich ist, betrachten wir das Resultat der ersten Titrierung nur als orientierenden Wert. Die gewonnenen Resultate sind in der folgenden Tabelle angeführt :

Bezeichnung	Stickstoffgehalt in %	»Essigsäure«-Gehalt		
		Gefunden I %	Gefunden II %	Berechnet %
Chitin (theoretisch)	5,91	—	—	25,31
»Gerüstsubstanz« 3	4,77	21,25	21,5	20,45
»Gerüstsubstanz« 4	4,13	20,90	17,9	17,70
»Gerüstsubstanz« 5	3,80	35,62	16,0	16,30

»Gefunden I« in der Tabelle bedeutet das ohne Permanganatbehandlung gewonnene Ergebnis, »Gefunden II« das nach der beschriebenen Permanganatbehandlung erhaltene Resultat.

Aus der Tabelle geht hervor, dass wir mit dieser Methode annähernd die errechneten Resultate erhielten (von der Annahme ausgehend, dass jedem Stickstoffatom eine Azetylgruppe entspricht). Die Reproduzierbarkeit der einzelnen Bestimmungen ist durchaus nicht einwandfrei, trotzdem fanden wir die Methode wegen ihrer Einfachheit und relativen Schnelligkeit für den gegebenen Zweck geeigneter als die angeführten Methoden der Literatur.

Es war anzunehmen, dass die nicht mit Lauge behandelten Verdauungsrückstände der natürlichen Gerüstsubstanz des Myzels näherstehen. Wir hatten zwei Muster, die 2,45% bzw. 2,44% Stickstoff enthielten, ferner auch eine nicht zu vernachlässigende Phosphormenge, nämlich 0,56%, während in den alkalisch behandelten Gerüstsubstanzen Phosphor höchstens in Spuren nachzuweisen war. Infolgedessen kann auch angenommen werden, dass ein Teil des Stickstoffs nicht an Essigsäure, sondern an Phosphorsäure gebunden ist ; ausserdem besteht auch die Möglichkeit, dass Azetyl an den stickstoffreien Zuckerteil gebunden vorkommt.

Leider wiesen die Werte des Azetylgehaltes der nicht mit Lauge behandelten Verdauungsrückstände eine derartige Streuung auf, dass sie quantitativ nicht bewertbar waren. Soviel konnten wir jedoch mit annehmbarer Wahrscheinlichkeit feststellen, dass der Azetylgehalt der Rückstände erheblich (mindestens um 50%) höher war, als auf Grund der Stickstoff-Äquivalente erwartet werden konnte. Die gewonnenen Werte schwankten im Vergleich zu den berechneten zwischen +150 und +200%.

Wir vermochten nicht festzustellen, worauf es zurückzuführen ist, dass der Azetylgehalt der nicht mit Lauge behandelten Verdauungsrückstände mit der beschriebenen Methode nicht bestimmt werden konnte. Auf Grund zahlreicher durchgeführter Versuche erscheint es jedoch als wahrscheinlich, dass in diesem Fall einerseits erheblich mehr Azetylgruppen anwesend sein müssen als in den mit Lauge behandelten Gerüstsubstanzen, anderseits diese Azetylgruppen bzw. ein Teil derselben mit den übrigen Bestandteilen der Gerüstsubstanz noch in anderer Verbindung stehen muss als in den vorher untersuchten Stoffen.

Zusammenfassung

Aus unseren Versuchen können wir folgende Schlussfolgerungen ziehen:

1. In erschöpfend alkalisch behandelten Gerüstsubstanzen entfällt auf jedes Stickstoffatom eine Azetylgruppe. Diese Feststellung scheint unzweifelhaft die Auffassung zu stützen, dass der Stickstoffgehalt der Gerüstsubstanz des Myzels — ähnlich wie beim tierischen Chitin — ausschliesslich oder in entscheidender Mehrheit mit dem Azetyl-Radikal in Verbindung steht.

2. Der Essigsäuregehalt des der alkalischen Behandlung nicht unterworfenen unverdauten Rückstandes ist erheblich höher als der aus dem Stickstoffgehalt (im Verhältnis 1 : 1) errechnete Wert und macht mindestens das Anderthalbfache und höchstens das Zweifache dieses Wertes aus. Hieraus kann zwar durchaus nicht mit Sicherheit der Schluss gezogen werden, dass der ganze Stickstoffgehalt azetyliert ist, doch wird dies hierdurch wahrscheinlicher gemacht. Als sicher ist jedoch anzusehen, dass ein Teil des Azetyls, und zwar mindestens ein Viertel und höchstens die Hälfte, nicht an Stickstoffatome gebunden ist.

3. Ein weiterer Teil der Gerüstsubstanz (mindestens 20% und höchstens 40%) besteht wahrscheinlich aus stickstoff- und azetylfreien Baustoffen, wahrscheinlich Kohlenhydraten.

4. Der Phosphatgehalt der Gerüstsubstanz ist in Wasser nicht löslich, wird aber bei Behandlung mit heisser Kalilauge vollständig abgespalten. Hieraus darf man folgern, dass sich der Phosphorgehalt der Gerüstsubstanz in Form von Phosphorsäureester an die Hydroxylgruppe eines Kohlenhydrats bindet; oder aber handelt es sich teilweise oder ganz um »unverdauliche« Phosphorproteidarten, die sich an die grossflächige Gerüstsubstanz adsorbieren; diese Annahme erscheint indessen unwahrscheinlich.

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ДАННЫЕ К ИЗУЧЕНИЮ СОСТАВА МИЦЕЛИЯ, ОБРАЗУЮЩЕГОСЯ ПРИ ПРОИЗВОДСТВЕ ПЕНИЦИЛЛИНА

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Резюме

Относительно состава мицелия образуемого грибком Penicillium chrysogenium имеются данные только о содержании в нем аминокислот и витаминов. Вместе с тем имеется интерес распознать и полисахарид, составляющий остов мицелия уже и потому, что вещество остова грибков вообще считают тождественным хитину, составляющему остов насекомых.

Из азотистых веществ свежепроизведенного мицелия с заторможенными энзиматическими функциями в крайнем случае половина может существовать в виде крупнейших молекул, а остальные уже автолизировались.

Для получения полисахаридной части в чистом виде авторы испробовали обработку мицелия методом, примененным Шоллем для выделения хитина уже в 1908 году. Однако и таким образом не могли получить определенное, воспроизводимое вещество, но стало несомненным, что вещество остова мицелия не может приниматься за взятый в старом смысле слова хитин, т. е. за вещество состоящее исключительно из азотистого ацетилглюкозамина, ибо в его построении принимают участие и безазотные соединения. Оказалось также, что вещество полученное сильнощёлочным методом Шолля, не сходно с естественным веществом остова, а является искусственным продуктом.

В результате исследования вещества остова, полученного не с помощью щёлочной обработки, а путем искусственного переваривания, стало вероятным, что все имеющиеся в нем азотистые соединения ацетилированы, но существует также и такая ацетиловая часть, которая сцепляется не с азотистыми соединениями, а с безазотными полисахаридами. Кроме того, вероятно, что вещество остова содержит также совершенно безазотные и безацетиловые углеводороды.

INFLUENZA IN SZEGED, 1953–54

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Early in December, 1953, a great number of influenza cases had been observed in Budapest and its environs [1]. Subsequently, the disease appeared also in Szeged, where from the throat washings of one of the early cases — obtained the 22nd December — we first succeeded in isolating the virus. In the further cases it was attempted to isolate the virus not only in fertile eggs but also in tissue cultures. As a result we have succeeded in isolating and identifying 9 influenza virus strains.

Methods

1. *Virus isolation.* Throat washings were made with physiological saline containing 1 per cent egg white, as protein in this concentration reduces the inactivation of the virus [2, 3]. The throat washings, after addition of 300 U/ml of penicillin and 300 μg/ml of streptomycin, were inoculated by means of a long sharp needle into the amniotic cavity of 12-day old embryonated eggs, held above a lamp. The inoculated eggs were maintained at 36° C for 3 days. Subsequently the amniotic fluids were harvested and, after pooling, inoculated again into the amniotic cavity of 12-day old embryonated eggs. Each throat washing was carried through three egg passages and the fluids haemagglutinating chicken or guinea pig red blood cells were selected for further passage.

Isolation of virus was attempted also in tissue cultures. To this purpose an apparatus was used [3], designed by one of us for titration of influenza virus infectivity. The tissue cultures, consisting of minced chorioallantoic membranes and nutrient fluid, were inoculated with 0,1 ml of the throat washing fluid containing antibiotics as described above. The cultures were incubated in a rotating drum at 37° C for three days. Further passages were made according to the principles mentioned. The fluid phase of the 5th tissue culture passage was inoculated into 12-day old embryonated eggs. Only materials containing virus — as revealed by the haemagglutination test — underwent further examination.

2. *Haemagglutination tests* were carried out essentially according to TAKÁTSY's method [4]. Instead of the original one stem loops we used double stemmed stunt loops which did not touch the walls of the holes in the titrating tray, and proved to be more stable. The bottom of the holes in the plexiglass titrating plates was not angular, but spherical in order to ensure a more complete mixing of the fluids by rotating the loops (Fig. 1).

As a diluent, physiological saline containing 1 per cent M/15 phosphate buffer of pH 7,2 was used. Under such conditions evaluation of the haemagglutination tests could be performed very accurately. The standard deviation calculated from numerous parallel tests could be diminished by adding to the diluent surface tension lowering substances (Table I).

Haemagglutination inhibition tests were carried out as follows. A twofold dilution series of serum was prepared by the aid of 0,025 ml spiral loops. Subsequently 4 haemagglutinating units of virus, in 0,025 ml volume, were added to each dilution. After allowing to stand at +4° C for 30 minutes, 0,025 ml of a 1 per cent suspension of chicken red blood cells was added. The mixtures were thoroughly shaken and the result was read after allowing them to stand again for 30 minutes at +4°C.

Table I

Standard deviations of parallel titrations in different diluents

Diluent	Number of the parallels	Mean	Standard deviation of the exponent
Saline	88	$2^{-7.7}$	$\pm$ 0,26
Tween 80 dil.: 10^{-5}	96	$2^{-7.6}$	$\pm$ 0,15
Tween 80 dil.: 10^{-4}	96	$2^{-7.4}$	$\pm$ 0,14

3. *Immune sera.* For immunization purposes chicken 5 to 6 weeks of age were used. They were first inoculated intravenously with 1 ml of the 11th egg passage material. The second inoculation was performed after 7 days in the same way. The animals were bled by cardiac puncture on the 20th day following the first vaccination. The sera were sealed in ampoules, stored at —15°C, and were inactivated by heating to 56°C for 30 minutes before use.

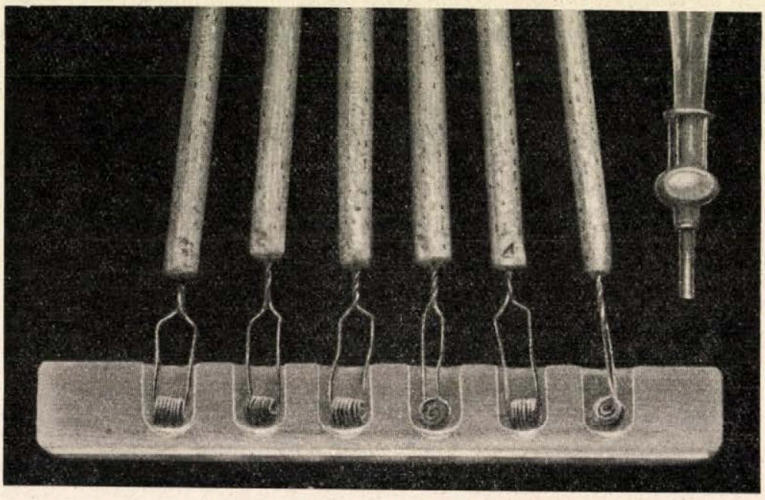

Fig. 1. Transverse section illustrating the position of the loops in the holes of the titrating plate. Right, Instillator, giving drops of 0,025 ml volume, prepared from an injection needle applicable to the end of a pipette (magnification 1 : 1,15).

Experimental

At the beginning of the influenza epidemic in Szeged in December, 1953, first only adults had been attacked by the disease ; after a few days some cases were observed also among children. Egg inoculation with throat washings from adult persons were performed at 6 instances and thus 4 strains were isolated. Isolation of virus from throat swabs obtained from sick children was attempted

Table II

Titration of the new influenza strains in tissue culture

Virus strains	Titration I			Titration II		
	Egg passage	ID_{50}/0,1 m_l	HA	Egg passage	ID_{50}/0,1 ml	HA
Sz 7/54	VIII.	10^5	>16	XII.	$10^{5.62}$	>16
Sz 8/54	VII.	$10^{6.5}$	>16	XII.	$10^{6.87}$	>16
Sz 9/54	IV.	10^4	14	XII.	$10^{5.32}$	>16
Sz 10/54	VII.	10^6	>16	XII.	$10^{5.62}$	>16
Sz 11/54	IV.	10^2	7	XII.	$10^{6.00}$	>16
Sz 12/54	IV.	10^4	7	XII.	$10^{6.50}$	>16
Sz 13/54	IV.^{x} + III.	10^3	8	IV.^{x} + XII.	$10^{6.25}$	>16
Sz 14/54	IV.^{x} + III.	10^3	2	IV.^{x} + XII.	$10^{5.83}$	>16
Sz 15/54	IV.^{x} + III.	10^2	2	IV.^{x} + XII.	$10^{7.00}$	14
Sz 16/54	IV.^{x} + III.	$10^{3.5}$	3	IV.^{x} + XII.	$10^{5.50}$	8

Sz = Szeged
HA = medians of the reciprocals of the haemagglutination titres
x = Tissue culture passages.

33 times. 24 freshly obtained swabs were separately washed into saline containing 1 per cent protein, and, after penicillin and streptomycin had been added, they were inoculated into 4 tissue culture ampoules each [5, 6]. As haemagglutination of chicken and guinea pig red blood cells by most of the tissue culture fluids proved to be dubious even after the 4th passage, only four samples were inoculated into the allantoic cavity of 12-day old embryonated eggs in order to demonstrate the presence of virus. Eggs inoculated with one of the culture fluids exhibited marked haemagglutination but of a low titre ; in the

other three fluids the titres ranged from 1 : 32 to 1 : 128. The virus strains thus isolated (Szeged 13/54, 14/54, 15/54, 16/54) were maintained by serial egg passages.

The problems of virus multiplication in tissue cultures will be dealt with in detail in another paper.

The infectivity of the allantoic fluid infected with the recently isolated virus strains was established in tissue cultures. Titration of infectivity was made with two samples obtained from different passages. The results are summarized in Table II.

The data presented in Table II show that strains isolated directly in embryonated eggs show a remarkable degree of infectivity and a definite haemagglutinating capacity. Strains isolated in tissue cultures yielded lower values in spite of their having been passaged 7 times (4 tissue culture and 3 egg passages). At the time of the 12th egg passage both the infectivity and haemagglutination titres were high. This indicates that our strains had originated from the sample obtained and not from a laboratory contamination. The virus causing the epidemic observed was isolated with relative ease, because out of the 7 clinical samples tested by inoculating embryonated eggs we have succeeded in isolating 5 strains. From one of the throat washing the virus was isolated not only by direct amniotic inoculation (Szeged 11/54) but also by injecting it into the allantoic cavity after preliminary tissue culture passages.

Identification of the strains was made by cross haemagglutination inhibition tests. The results presented in Table III are expressed by the fractions calculated according to the principles given by the World Influenza Centre [7], and by the «d» values suggested by the State Institute of Hygiene, Budapest [8].

It is seen from Table III that all the recently isolated strains were of the A prime type and were essentially different from the PR8 and Lee strains. They were closely related to the Sweden 3/50 and Paris PL 1/49 strains. Strains Szeged 11/54 and Szeged 14/54 had fairly similar properties, since they both had been isolated from the same clinical sample by amniotic and tissue culture inoculation, respectively. It is also noteworthy that strains Szeged 15/54 and Szeged 16/54 exhibited properties somewhat different from those of the other recently isolated strains.

Summary

During the 1953—54 influenza epidemic observed in Szeged 9 strains of influenza virus have been isolated. Out of these, 4 had previously been cultivated in minced chorioallantoic membrane tissue cultures through 5 passages and subsequently maintained in the allantoic cavity of fertile eggs. All the strains isolated vere of the A prime type and were antigenically related to the Sweden 3/50 and Paris P. L. 1/49 virus strains.

Table III

Comparison of the influenza strains on the basis of cross haemagglutination inhibition test

Virus strains	Sz* 7/54	Sz 8/54	Sz 9/54	Sz 10/54	Sz 11/54	Sz 12/54	Sz 13/54	Sz 14/54	Sz 15/54	Sz 16/54	Sweden 3/50	Paris PL 1/49	PR 8	Lee
Sz 7/54		1 1,63	1 1,69	1 1,56	1 1,00	1 1,31	1 2,00	1 1,10	1 5,23	1 4,25	1 1,31	1 1,85	1 49,8	1 13,9
Sz 8/54	6		1 1,95	1 1,69	1 1,79	1 1,91	1 2,14	1 2,14	1 3,26	1 7,15	1 2,14	1 2,71	1 81,2	1 32,0
Sz 9/54	6	8		1 2,18	1 1,74	1 1,83	1 2,62	1 2,68	1 4,00	1 5,24	1 2,32	1 2,98	1 71,5	1 36,6
Sz 10/54	5	6	9		1 2,00	1 1,79	1 1,28	1 2,00	1 4,27	1 7,40	1 2,32	1 1,12	1 91,0	1 50,8
Sz 11/54	0	7	6	8		1 1,10	1 2,42	1 1,85	1 2,45	1 4,80	1 1,71	1 1,65	1 35,2	1 12,7
Sz 12/54	3	7	7	7	1		1 2,34	1 1,51	1 1,64	1 6,20	1 1,63	1 2,26	1 64,0	1 22,6
Sz 13/54	8	9	11	3	10	10		81 1,20	1 3,70	1 4,55	1 3,03	1 1,79	1 35,2	1 19,7
Sz 14/54	1	9	11	8	7	5	2		1 1,20	1 3,60	1 2,11	1 1,96	1 35,8	1 11,6
Sz 15/54	19	14	16	17	10	6	15	2		1 5,55	1 1,92	1 2,26	1 45,2	1 14,6
Sz 16/54	17	23	19	23	18	21	17	15	20		1 9,38	1 3,20	1 19,8	1 11,0
Sweden 3/50	3	9	10	10	6	6	14	9	7	26		1 2,47	1 181	1 64,0
Paris PL 1/49	7	12	12	1	6	9	7	8	9	13	10		1 181	1 71,5
PR8	45	51	50	52	41	49	41	41	44	35	59	59		1 71,5
Lee	30	40	42	45	30	36	34	28	31	28	48	49	49	

* Szeged

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ЭПИДЕМИЯ ГРИППА, ПОЯВИВШАЯСЯ В г. СЕГЕДЕ ЗИМОЙ 1953–54 гг.

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Р е з ю м е

Во время эпидемии гриппа, появившейся в Сегеде зимой 1953–54 гг., авторы изолировали 9 штаммов гриппозного вируса. Из этих штаммов четыре были культивированы сначала на размельченной тканевой культуре хорионаллантоидной оболочки, затем, после 5-го пассажа сохранялись в аллантоидной полости эмбрионированного куриного яйца. Все выделенные штаммы относились к группе A' и по антигенной структуре близки гриппозным вирусам Sweden 3/50 и Paris PL 1/49.

BEHAVIOUR OF SALMONELLA STRAINS IN SYNTHETIC MEDIA

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The nutritional requirements of different species of *Salmonella* was examined in series of synthetic media. The test strains were those most frequently occurring in veterinary practice.

Materials and methods

The basal medium consisted of NaCl 8 g, KH_2PO_4 1 g, $Na_2S_2O_3$ 0,1 g, $MgSO_4$ 0,1 g bisdistilled water 1000 g. The ammonium sulphate and the amino acids, which served as source of N, were added to the basal medium in concentrations of 0,001 M. The substances supplying C and energy, such as glucose, succinic acid, fumaric acid and sodium citrate, were added in 0,5 per cent concentrations. With the exception of glucose, all the ingredients added to the basal medium were sterilized together with the medium. After sterilization the medium was adjusted to pH 7,2 with NaOH. The glucose solution was sterilized by filtration and added to the sterilized medium. The following amino acids were added to the synthetic media: dl-alanine, dl-serine, l-cysteine, l-cystine, l-tyrosine, dl-tryptophane, l-asparagine, l-glutamic acid, dl-methionine, dl-threonine, dl-valine, dl-leucine, dl-isoleucine, l-arginine, dl-histidine, and dl-lysine. The different media were so composed that each of them contained only one of the above amino acids, with or without ammonium sulphate. To the amino acid media neither glucose, nor organic acids were added. Glucose, lactic acid, succinic acid, fumaric acid and sodium citrate were examined one by one, in basal media containing ammonium sulphate. Of the media so prepared volumes of 5 ml were distributed into sterile test tubes. These were exposed to a 48-hour sterility test and then inoculated with suspended and twice washed organisms from a 18-hour agar slant culture. Microbial density was adjusted to 10^7 organisms per ml in a Pulfrich nephelometer. The media were inoculated with 0,1 ml of the suspension (approximately 10^6 microbes) and then incubated for 96 hours at 37°C. At intervals of 24 hours the rate of bacterial growth was examined by determining by nephelometry the changes in turbidity. The strains were then identified by transfer to agar plates and by serological methods.

Laboratory strains and recently isolated strains of *Salmonella* were examined. The strains were identified on the basis of carbohydrate breakdown and by serological tests. The total number of strains tested was 23, These included: *S. paratyphi A* 1 strain, *S. typhimurium* 1, *S. abortus-equi* 1, *S. abortus-ovis* 1, *S. cholerae-suis var.* Kunzendorf 11, *S. typhi-suis var.* Voldagsen 10, *S. gallinarum* 7, and *S. enteritidis* Gärtner 1 strain.

Results

In the first part of this work synthetic media were inoculated with representative laboratory strains. The results of the tests, which were repeated several times, are shown in Table I.

The data in Table I reveal that the strains of Salmonella tested grew only in media containing the asparagine, glutamic acid, serine, histidine, alanine,

Table I

Behaviour of species of Salmonella in synthetic media

Species of Salmonella tested	Basal medium																Basal medium + Ammonium sulphate +				
	dl-α-alanine	dl-serine	l-cysteine	l-cystine	dl-methionine	dl-threonine	dl-valine	dl-leucine	dl-isoleucine	l-tyrosine	dl-tryptophane	dl-histidine	l-asparagine	l-glutamic acid	l-arginine	dl-lysine	Glucose	Succinic acid	Fumaric acid	Lactic acid	Sodium citrate
S. paratyphi A	—	200	50	—	—	—	—	—	—	—	—	—	—	200	—	—	200	60	—	200	—
S. typhi-murium	160	330	—	—	—	—	—	—	—	—	—	250	200	200	—	—	330	500	500	1000	400
S. abortus-equi	330	200	—	—	—	—	—	—	—	—	—	200	250	330	—	—	400	500	500	1000	500
S. abortus-ovis	330	330	50	—	—	—	—	—	—	—	—	330	100	250	—	—	250	1000	500	1000	500
S. cholerae-suis var. Kunzendorf	70	200	50	—	—	—	—	—	—	—	—	200	200	200	—	—	200	200	200	300	—
S. typhi-suis var. Voldagsen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—
S. enteritidis Gärtner ...	330	250	—	—	—	—	—	—	—	—	—	500	160	250	—	—	250	1000	1000	1000	—
S. gallinarum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

The values are those of relative turbidity, as determined in a Pulfrich nephelometer, using a 4/E filter.

and partly cysteine. As to the other amino acids tested, these did not support the growth of the organisms even in the presence of ammonium sulphate. In the presence of ammonium sulphate and glucose, or in that of organic acids the strains differed in behaviour.

The data in Table I call attention to the differences between *S. cholerae-suis var.* Kunzendorf and *S. typhi-suis var.* Voldagsen. The Voldagsen strain could namely utilise none of the amino acids for growth, while the Kunzendorf-type strain grew well in media containing serine, histidine, asparagine and glutamic acid, respectively.

The behaviour of *S. gallinarum* was also remarkable. This organism grew neither in media containing amino acids, nor in those containing ammonium sulphate, glucose or lactic acid. These three species of Salmonella, which frequently occur in veterinary practice in Hungary, were then subjected to further studies, testing strains of *S. cholerae-suis var.* Kunzendorf, *S. typhi-suis var.* Voldagsen and *S. gallinarum*, freshly isolated in different parts of the country.

Table II includes data illustrative of differences between strains. Media that failed to support growth of any of the test strains have been ignored.

An analysis of the data in Table II reveals that, among the strains of Salmonella tested, those of *S. gallinarum* were the most pretentious since these did not grow in any of the synthetic media. The strains of *S. cholerae-suis* were uniform in that serine, glutamic acid, or ammonium sulphate with glucose or lactic acid all supported their growth. Differences occurred in the utilisation of the other amino acids mentioned, such as alanine, asparagine, histidine, as it was persistently recorded on reproducing the tests. This phenomenon appears to indicate that the strains belonging to the Kunzendorf type of *S. cholerae-suis* may differ in biochemical activity with respect to amino acids. For example, strain Kh. 11, thought to be of the Kunzendorf type on the basis of carbohydrate breakdown, was entirely different from the Kunzendorf in its behaviour in synthetic media, in which respect it was more similar to strains of the Voldagsen type. According to the trypaflavine test, this strain was of the R-type. It is believed that the divergent mode of amino acid utilisation may be attributed to the R property. Similar findings have been reported by LEVINE et al. for the R variant of *Malleomyces pseudomallei* [8]. The *S. typhi- suis var.* Voldagsen strains tested were biochemically far less active on amino acids than strains of the Kunzendorf type. Most of the former failed to grow in media containing amino acid and could utilise ammonium sulphate only in the presence of lactic acid. Certain strains (K_3, K_4) of the Voldagsen type grew also in the presence of glutamic acid, indicating that variations may occur within this type, too. All the Voldagsen strains grew abundantly in the presence of glutamic acid and glucose.

Table II

Behaviour of Salmonella cholerae-suis var. Kunzendorf, S. typhi-suis var. Voldagsen and Salmonella gallinarum strains in synthetic media

Species of Salmonella tested		Basal medium							Basal medium + Ammonium sulphate +					
			dl-α-alanine	dl-serine	l-cysteine	dl-histidine	l-asparagine	l-glutamicacid		Glucose	Succinic acid	Fumaric acid	Lactic acid	Sodium citrate
Salmonella cholerae- suis var. Kunzendorf		—	70	200	50	200	200	200	—	200	200	200	300	—
	(D. 1.)	—	—	100	—	50	—	100	—	100	—	—	300	—
	(D. 3.)	—	—	120	—	120	—	200	—	50	—	—	200	—
	(D. 4.)	—	—	100	—	100	50	200	—	50	100	—	300	—
	(D. 5.)	—	—	100	—	80	—	120	—	56	70	—	500	—
	(D. 6.)	—	—	100	—	80	—	80	—	50	—	—	300	—
	(D. 8.)	—	—	70	—	—	50	200	—	50	80	—	500	—
	(D. 9.)	—	—	100	—	100	—	100	—	50	—	—	300	—
	(D. 17.)	—	—	150	60	100	—	130	—	100	90	—	500	—
	(Kh 10.)	—	—	100	50	200	100	200	—	200	200	200	500	—
	(Kh 11.R.)	—	—	—	—	—	—	—	—	—	—	—	200	—
Salmonella typhi-suis var. Voldagsen	(Bp. XX)	—	—	—	—	—	—	—	—	—	—	—	100	—
	(B. 1.)	—	—	—	—	—	—	—	—	—	—	—	100	—
	(B. 2.)	—	—	—	—	—	—	—	—	—	—	—	130	—
	(B. 3.)	—	—	—	—	—	—	—	—	—	—	—	100	—
	(K. 8.)	—	—	—	—	—	—	—	—	50	—	—	100	—
	(D. 2.)	—	—	—	—	—	—	—	—	50	—	—	100	—
	(D. 7.)	—	—	—	—	—	—	—	—	—	—	—	100	—
	(D. 16.)	—	—	—	—	—	—	—	—	50	—	—	120	—
	(K. 3.)	—	—	—	—	—	—	100	—	—	—	—	100	—
	(K. 4.)	—	—	—	—	—	—	100	—	—	—	—	100	—
Salmonella gallinarum		—	—	—	—	—	—	—	—	—	—	—	—	—
	(D. 11.)	—	—	—	—	—	—	—	—	—	—	—	—	—
	(D. 13.)	—	—	—	—	—	—	—	—	—	—	—	—	—
	(K. 6.)	—	—	—	—	—	—	—	—	—	—	—	—	—
	(K. 11.)	—	—	—	—	—	—	—	—	—	—	—	—	—
	(K. 14.)	—	—	—	—	—	—	—	—	—	—	—	—	—
	(K. 15.)	—	—	—	—	—	—	—	—	—	—	—	—	—

The values are those of relative turbidity, as determined in a Pulfrich nephelometer, using a 4/E filter.

Discussion

Experiments in synthetic media have shown that the Kunzendorf and Voldagsen type strains, although identical in serological behaviour, possess different enzyme systems, as manifested with divergences in action on amino acids, and, in part, on ammonium sulphate and glucose, or on the organic acids tested. The different enzyme system of *S. suipestifer* strains is reflected by a variability of biochemical activities, their variability being probably the explanation of the difference in clinical and pathological patterns observable in patients. FORMAL [3], HEPLAR et al. [4] assume a close relationship between biochemical activity and pathogenicity within the same bacterium species. The results of the experiments show clearly the role of amino acids in the metabolism of Salmonella. In communications concerned with the metabolism of Salmonella, the amino acids are usually referred to as supplements which must be added to the medium, because the bacterium is incapable of synthesizing them from the inorganic source provided by the medium (LEDERBERG [7], PLOUGH et al. [10], WARE [11], DAVIS et al. [1], JONES and associate [15]). Our findings appear to indicate that under the experimental conditions employed the amino acids supporting the growth of Salmonella do not serve as substitutes for the lacking amino acid synthesis, but they by themselves are also capable of providing the bacterium with the N and C required for its growth. A given strain of Salmonella is either capable or incapable of oxidizing the molecules of the amino acid so as to utilise N and C derivable from it. For example, the Kunzendorf strains grew also in media containing only glutamic acid, in which media the Voldagsen-type strains generally did not grow. When lactic acid or glucose had been added to the medium containing glutamic acid, the Voldagsen-type bacteria, too, could grow. From glutamic acid the Voldagsen-type strain can utilize only the nitrogen and it is from glucose or lactic acid that it has to derive the energy required for its growth.

It is remarkable that some of the *S. cholerae-suis var.* Kunzendorf strains failed to grow in media containing both ammonium sulphate and histidine, while media containing histidine but no ammonium sulphate supported the growth of this organism. In the former case the ammonium sulphate inhibited the oxidation of histidine by the bacterium.

Amino acid breakdown by bacteria is dependent on the function of bacterial dehydrogenase and decarboxylase. The differences in action on amino acids may be explained on the basis of differences in these enzymes. KAUFFMANN and MØLLER [6] found differences in the decarboxylase activity of different Salmonella strains. They examined amino acid decarboxylation under anaerobic conditions in peptone-broth media, and found that the strains of Salmonella could break down lysine and arginine, but not glutamic acid. In our investigations, in which one given amino acid was the sole source of energy and nitrogen

n the synthetic medium, the Salmonella strains tested decomposed glutamic acid, but failed to break down lysine and arginine. The divergence between our own results and those of KAUFFMANN and MØLLER may be attributed to differences in experimental conditions.

Summary

The nutritional requirements of Salmonellae were investigated on media containing inorganic salts and as N and energy source one amino acid.

Only four of the amino acids tested (notably glutamic acid, serine, histidine and asparagine) have been found to be capable of supplying sufficient N and C to Salmonellae. The single species of Salmonella were not uniform as concerns utilization of these amino acids. *S. cholerae-suis var.* Kunzendorf strains grew abundantly in media containing any one of the above amino acids, while these media did not support the growth of either the *S. typhi-suis var.* Voldagsen, or *S. gallinarum* strains.

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ПОВЕДЕНИЕ ШТАММОВ SALMONELLA НА СИНТЕТИЧЕСКИХ ПИТАТЕЛЬНЫХ СРЕДАХ

Ф. МУРАНЬИ и Л. ПРОХАСКА

Р е з ю м е

Авторы исследовали обмен веществ штаммов Salmonella с применением синтетических питательных сред. Употребляли такие синтетические питательные среды, в которых в качестве источников азота и энергии добавляли по одной аминокислоте. Рост штаммов Salmonella на изготовленных таким образом серийных питательных средах демонстрируют таблицы I и 2. Поведение типов Salmonella по отношению к различным аминокислотам было различно.

Наиболее требовательны штаммы Salmonella gallinarum, так как для размножения не могли использовать ни одной из исследуемых аминокислот. Определимые различия были обнаружены между штаммами Salmonella cholerae suis var. Kunzendorf и Salmonella typhi suis var. Voldagsen, паразитирующими на свиньях. Штаммы типа Kunzendorf развиваются на средах содержащих гистидин, серин или глютаминовую кислоту. В противоположность этому штаммы типа Voldagsen не размножаются на упомянутых средах. Биохимическая активность исследуемых штаммов Salmonella по отношению к аминокислотам в повторных опытах всегда была одинакова.

UNTERSUCHUNGEN ÜBER DIE TYPHUS-INTRAKUTANPROBE

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Infolge der systematischen Massenimpfungen gegen Typhus kommen in Ungarn Typhuserkrankungen selten vor. Doch treten manchmal schwere Impfreaktionen auf, ja es scheint, als ob sich ihre Zahl in den letzten Jahren vermehrt hätte. Eine Klärung der Frage, ob beim Zustandekommen der schweren Typhus-Impfreaktionen ausser der toxischen Eigenschaft des Impfstoffes nicht auch die Überempfindlichkeit der geimpften Individuen eine Rolle spielt, ist daher von wesentlicher Bedeutung.

Der in Ungarn verwendete Typhus-Impfstoff wurde von LOVREKOVICH und RAUSS ausgearbeitet [1] und dann von RAUSS modifiziert [2]. Der Impfstoff ist ein an Aluminiumhydroxyd-Gel adsorbierter, mit Trichloressigsäure behandelter Extrakt von Typhusbakterien. Um die Natur der Impfreaktion festzustellen, muss also untersucht werden, ob sich eine Überempfindlichkeit des Organismus gegenüber den mit Trichloressigsäure behandelten Typhusbakterien nachweisen lässt. Zur Klärung der Frage wandten wir die Intrakutanprobe an.

Die Literaturangaben über die Natur der Typhus-Intrakutanprobe sind nicht einheitlich. Die Resultate der meisten mit der Typhus-Intrakutanprobe vorgenommenen Untersuchungen deuten auf den allergischen Charakter der Probe hin (GLOUKHOFF und Mitarbeiter [3]; SELENIN, [4]; COSTA und Mitarbeiter, [5]; ALISOW und MOROSCHKIN [6]; KENDRICK [7]). Aus den Untersuchungen von KRESTOVNIKOWA wiederum kann auf die toxische Natur der Probe geschlossen werden. Die erwähnten Autoren hatten die Intrakutanprobe mit dem Filtrat von Typhuskulturen oder mit Typhusbakterien bzw. mit deren Autolysat vorgenommen. Bei unseren eigenen Untersuchungen führten wir die Intrakutanproben mit einem unter Anwendung von Trichloressigsäure hergestellten Extrakt der Typhusbakterien durch, d. h. mit jenem Stoff, der in dem in Ungarn gebräuchlichen RAUSSschen präzipitierten Typhus-Impfstoff enthalten ist. Von der Verdünnung 1 : 10 000 des Extraktes injizierten wir 0,1 ml in die Haut der Beugeseite des Unterarms. Die Reaktionen wurden nach 24 Stunden kontrolliert. Als positives Resultat galt eine Hautrötung von 10 mm oder grösserem Durchmesser.

Mit dem unter Anwendung von Trichloressigsäure hergestellten Typhusbakterien-Extraktstoff nahmen wir intrakutane Impfungen an verschiedenen Altersgruppen vor. Mit diesen Untersuchungen wollten wir die Natur der Typhus-Intrakutanprobe klären. Die Positivität der allergischen Hautproben (z. B. der Tuberkulinprobe) nimmt nämlich mit dem Alter zu, während die Positivität der Hautproben vom Toxin-Antitoxintyp (SCHICKsche, DICKsche Probe) mit dem Alter abnimmt.

Wir impften insgesamt 656 gesunde Personen verschiedener Altersgruppen intrakutan. Die Resultate der Hautproben sind — nach Altersgruppen geordnet — aus Tabelle I ersichtlich.

Tabelle I

Ergebnisse der Typhus-Intrakutanproben bei gesunden Individuen nach Altersgruppen

		Insgesamt	15—19	20—29	30—59	über 60
			Jahre alte Individuen			
Anzahl der Untersuchten		656	340	108	195	13
Positiv	Anzahl	188	47	28	104	9
	%	29	14	26	53	70
				*	**	

	χ^2	P%
*	7,77	<1
**	20,1	<0,1

Aus den Angaben der Tabelle I geht hervor, dass die Positivität der Typhus-Intrakutanprobe mit dem Alter steigt. Die Differenzen zwischen der ersten und zweiten sowie der zweiten und dritten Altersgruppe sind stark signifikant. Auch in der vierten Altersgruppe ist die nahezu gleiche prozentuale Erhöhung der positiven Fälle zu beobachten, wenn sich auch im Hinblick auf die geringe Zahl der untersuchten Fälle hier keine Signifikanz feststellen lässt.

Die mitgeteilten Angaben erweisen, dass sich mit zunehmendem Alter dem unter Anwendung von Trichloressigsäure hergestellten Typhusbakterienextrakt gegenüber eine allmählich steigende Überempfindlichkeit entwickelt.

Die Typhus-Intrakutanprobe nahmen wir gleichzeitig auch an 546 Geisteskranken vor. Bekanntlich ist die allergische Reaktionsbereitschaft der Geisteskranken sehr gering (MCALLISTER und HECKER [9]; LEAVITT [10]; MOLHOLM [11]. Dementsprechend war zu erwarten, dass die Typhus-Intrakutanprobe bei Geisteskranken in niedrigerem Prozentsatz positiv ausfallen würde als bei gesunden Individuen. Die Resultate der an Geisteskranken durchgeführten Untersuchungen sind auf Tabelle II zusammengefasst.

Tabelle II

Ergebnisse der Typhus-Intrakutanproben bei Geisteskranken nach Altersgruppen

		Insgesamt	15—19	20—29	30—59	über 60
			Jahre alte Individuen			
Anzahl der Untersuchten		546	24	63	364	95
Positiv	Anzahl	201	5	22	138	36
	%	37	21	35	38	38

Tabelle II zeigt, dass positive Typhus-Intrakutanproben unter den verschiedenen Altersgruppen der Geisteskranken ungefähr im gleichen Prozentsatz vorkommen. Lediglich zwischen den ersten beiden Altersgruppen ist eine geringe Differenz festzustellen, die sich jedoch statistisch nicht als signifikant erwies. Bei den älteren Altersgruppen der Geisteskranken liegt die Positivität der Typhus-Intrakutanprobe ungefähr auf dem gleichen niedrigen Niveau. Die Gestaltung der positiven Typhus-Intrakutanproben bei gesunden Individuen und Geisteskranken nach dem Alter geht aus Abb. 1 anschaulich hervor.

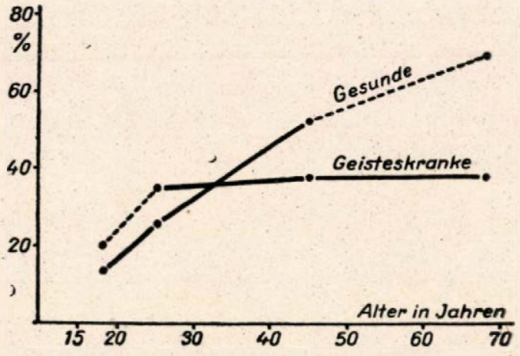

Abb. 1. Die Positivität der Typhus-Intrakutanprobe bei gesunden Individuen und Geisteskranken nach dem Alter

Nach diesen Resultaten zeugen unsere Untersuchungen an Geisteskranken ebenfalls für den allergischen Charakter der Typhus-Intrakutanprobe.

Im Zeitpunkt der Intrakutanproben wurde von 351 gesunden Personen zwecks Bestimmung des Typhus-O-Agglutinintiters auch Blut entnommen. Die Ergebnisse der Blutuntersuchungen sind auf Tabelle III angeführt.

Die Angaben der Tabelle III zeigen, dass der Typhus-O-Agglutinintiter der positive und negative Typhus-Intrakutanreaktion ergebenden gesunden Personen annähernd identisch war Zwischen den Resultaten der Hautprobe und den aktuellen homologen Antikörpertitern besteht also kein Zusammenhang.

Mehrere Autoren hatten untersucht, ob aus den Ergebnissen der einige Tage vor der Typhus-Schutzimpfung vorgenommenen Intrakutanprobe auf die zu erwartende Stärke der Impfreaktion Schlüsse gezogen werden können. WOLFF-

Tabelle III

Vergleich der O-Agglutinintiter mit den Ergebnissen der Intrakutanproben

Ergebnisse der Intrakutanprobe	Anzahl der Untersuchten	Durchschnittlicher O-Agglutinintiter
Positiv	150	1 : 26
Negativ	201	1 : 30
Insgesamt	351	1 : 28

EISNER [12] sowie SOHIER und Mitarbeiter [13] hatten zwischen der Stärke der Intrakutanprobe und der Schwere der Impfreaktion entschiedene Parallelität festgestellt, während die Untersuchungen von MAASEN und KAUDE [14] keinen engen Zusammenhang zeigten. Vorstehende Autoren hatten die Intrakutanproben mit verschiedenen Verdünnungen der zur Schutzimpfung benutzten bazillenhaltigen Vakzine durchgeführt. Diese Frage wurde auch von uns untersucht. An 174 Personen nahmen wir mit der Verdünnung 1 : 10 000 des mit Trichloressigsäure hergestellten Typhusbakterienextraktes die Intrakutanprobe vor und impften sie dann am folgenden Tage mit 1,0 ml des aus dem gleichen Extraktstoff bereiteten RAUSSschen präzipitierten Impfstoffs. Der Zusammenhang zwischen den Resultaten der Intrakutanprobe und den lokalen Impfreaktionen geht aus Tabelle IV hervor.

Tabelle IV

Zusammenhang zwischen den Ergebnissen der Intrakutanproben und Impfreaktionen

Ergebnis der Intrakutanprobe	Anzahl der untersuchten Personen	Hiervon					
		keine Infiltration		1—4 cm grosse Infiltration		5 cm grosse oder grössere Infiltration	
		Anzahl	%	Anzahl	%	Anzahl	%
Negativ	136	28	21	95	70	13	* 9
Positiv	38	2	5	26	68	10	* 27
Insgesamt	174	30	18	121	69	23	13
		χ^2		P (%)			
		* 5,88		<2			

Den Angaben der Tabelle ist zu entnehmen, dass nach positiver Intrakutanprobe schwere Infiltrationen (mit 5 cm oder grösserem Durchmesser) dreimal häufiger vorkommen als nach negativen Intrakutanreaktionen. Eine gewisse Parallelität ist also zu beobachten, doch besteht kein absoluter Zusammenhang.

Tabelle V

Zusammenhang zwischen dem Ergebnis der Intrakutanproben und Impfreaktionen
Allgemeinreaktionen

Intrakutanprobe	Temperatur 37,0—37,9°		Temperatur 38,0—39°		Kopfschmerzen		Untersuchte Personen	
	Anzahl	%	Anzahl	%	Anzahl	%	Anzahl	%
Negativ	8	6	3	2	22	16	136	100
Positiv	5	13	2	5	11	30	38	100
Insgesamt	13	8	5	3	33	19	174	100

Wir kontrollierten auch die Allgemeinreaktionen der Geimpften. Die diesbezüglichen Resultate sind auf Tabelle V zusammengefasst.

Aus Tabelle V ist ersichtlich, dass Fieber und Kopfschmerzen nach positiver Intrakutanprobe auf Wirkung des präzipitierten Impfstoffs doppelt so häufig vorkamen wie nach der negativen Probe.

Besprechung

Bei unseren Untersuchungen konnten wir feststellen, dass die Positivität der mit dem unter Anwendung von Trichloressigsäure hergestellten Typhusbakterienextrakt vorgenommenen Intrakutanproben mit dem Alter zunimmt. Einen ähnlichen Zusammenhang zwischen den Resultaten der Typhus-Intrakutanproben und dem Alter hatten bereits SOHIER und Mitarbeiter beschrieben [15]. Diese Autoren hatten jedoch die Intrakutanprobe mit verdünnter bazillenhaltiger Vakzine ausgeführt. Eines der Ziele unserer Untersuchungen war die Feststellung, ob auch dem mit Trichloressigsäure hergestellten Extraktstoff der Typhusbakterien gegenüber eine mit dem Alter zunehmende Überempfindlichkeit nachgewiesen werden kann. Da unsere Untersuchungen in dieser Frage ein positives Resultat ergaben, ist als wahrscheinlich anzusehen, dass bei den Impfreaktionen des bei uns gebräuchlichen, trichloressigsauren Extraktstoff enthaltenden RAUSSschen präzipitierten Typhus-Impfstoffes auch ein allergischer Faktor eine Rolle spielt.

Bei der Untersuchung der positiven Typhus-Intrakutanproben der Überempfindlichkeitssymptome selten aufweisenden und nur über geringe allergische Reaktionsbereitschaft verfügenden Geisteskranken konnte festgestellt werden, dass die intrakutane Positivität der zur jüngsten Altersgruppe gehörenden Geisteskranken kaum von den Resultaten der entsprechenden gesunden Altersgruppe abweicht. Vom 20. Jahr an folgt jedoch die Kurve der positiven Typhus-Intrakutanproben der Geisteskranken nicht der bei Gesunden fest-

gestellten Kurve, sondern bleibt in allen anderen Altersgruppen beinahe vollständig auf dem gleichen Niveau, was darauf hindeutet, dass es zur Verminderung der allergischen Reaktionsbereitschaft von Geisteskranken einer gewissen Zeit bedarf. Diese Ergebnisse bestärken unsere Feststellungen über die allergische Natur der Typhus-Intrakutanprobe.

Die Typhus-O-Agglutinintiter der positive und negative Typhus-Hautproben ergebenden gesunden Personen waren zur Zeit der Hautproben annähernd gleich. Zwischen dem Ausmass der Allergie gegenüber dem Typhusextraktstoff und dem aktuellen homologen Antikörpertiter konnten wir also keinen Zusammenhang feststellen. Es ist indessen anzunehmen, dass der dem Typhusantigen gegenüber überempfindliche Organismus nicht nur auf die Einführung des Typhusantigens in die Haut in gesteigertem Masse reagiert, sondern auch in der homologen Antikörperproduktion höhere Reaktivität aufweist. Hierauf deuten unsere Beobachtungen, wonach der durchschnittliche O-Agglutinintiter der lokale Infiltration aufweisenden Personen zwei Wochen nach der Impfung signifikant höher ist als der im gleichen Zeitpunkt untersuchte durchschnittliche Titer reaktionsfreier Individuen [16]. Die Vermeidung schwerer Reaktionen nach Typhusimpfungen wäre in gewissen Fällen (Schwangere, Herzkranke usw.) besonders wichtig. Dieses Problem taucht im Zusammenhang mit einer Impfung der Umgebung oder Massenimpfungen gegen Typhus häufig auf. In derartigen Fällen wäre ein Verfahren von Nutzen, mit dessen Hilfe auf die Reaktion nach der Typhusimpfung Schlüsse gezogen werden können. Hinsichtlich einer diesbezüglichen Brauchbarkeit der Intrakutanprobe sind die Literaturangaben widersprechend. Aus den Resultaten der Intrakutanprobe, die wir mit trichloressigsaurem Typhusbakterienextraktstoff vornahmen, konnten wir nicht mit Sicherheit folgern, welche Reaktionen der aus dem identischen Extraktstoff hergestellte präzipitierte Impfstoff auslösen wird. Aus unseren Untersuchungen lässt sich lediglich feststellen, dass nach negativer Probe auf Wirkung des präzipitierten Impfstoffs höchstens in 10% der Fälle eine schwere lokale Impfreaktion zu erwarten und die Wahrscheinlichkeit einer Allgemeinreaktion geringer ist als nach positiver Intrakutanprobe. Zwischen dem Resultat der Intrakutanprobe und der Stärke der Impfreaktion besteht vielleicht deshalb kein genauer Zusammenhang, weil das Präzipitat die Resorptionsverhältnisse des Antigens verändert. Es muss aber auch daran gedacht werden, dass die Reaktivität des Organismus gegenüber dem Typhusantigen durch die vor der Impfung ausgeführte Intrakutanprobe beeinflusst wird.

Zusammenfassung

656 gesunde Individuen wurden mit trichloressigsaurem Extraktstoff von Typhusbakterien intrakutan geimpft. Die Positivität der Intrakutanprobe nahm mit dem Alter zu. Zu gleicher Zeit wurde die Intrakutanprobe auch an

546 Geisteskranken vorgenommen. Die Positivität der Typhus-Intrakutanproben der allergischen Krankheiten gegenüber im allgemeinen weniger empfänglichen Geisteskranken war nur in den beiden jüngsten Altersgruppen erhöht und blieb bei den älteren Altersgruppen auf dem gleichen niedrigen Niveau.

Der durchschnittliche Typhus-O-Agglutinintiter war im Zeitpunkt der Probe bei den intrakutan positiven und negativen gesunden Individuen identisch.

Auch die Resultate der Intrakutanproben und die auf Wirkung des 24 Stunden nach der Probe subkutan verabreichten präzipitierten Typhus-Impfstoffs eintretenden Impfreaktionen wurden verglichen. Ein enger Zusammenhang zwischen diesen war nicht zu beobachten. Es liess sich lediglich feststellen, dass nach negativer Intrakutanprobe nur in 10% der Fälle eine schwere lokale Impfreaktion zu erwarten und auch die Wahrscheinlichkeit einer Allgemeinreaktion geringer ist als nach positiver Intrakutanprobe.

Für ihre freundliche Hilfe bei unseren Untersuchungen danken wir auch an dieser Stelle Frau L. GYIMES, Direktor der Staatlichen Heilanstalt für Nerven- und Geisteskranke, ferner allen Mitarbeitern dieses Instituts und Betriebsarzt L. CSÁKTORNYAI sowie für die statistische Auswertung der Resultate GY. BARSY.

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ИССЛЕДОВАНИЯ В СВЯЗИ С ТИФОЗНОЙ ВНУТРИКОЖНОЙ ПРОБОЙ

ДЬ. ХЕДЬЕШШИ и Ш. БОЖОКИ

Р е з ю м е

Авторы прививали внутрикожно 656 здоровых лиц трихлоруксусным экстрактом тифозных бактерий. Положительность внутрикожных проб повышалась с возрастом. Параллельно производили внутрикожные пробы также и на 546 психотических больных. У психотических больных — которые являются вообще менее восприимчивыми к аллергическим болезням — положительность внутрикожных проб повышалась с возрастом только в двух наиболее молодых возрастных группах, в старших группах оставалась на одинаковом уровне.

Средний титр О-агглютинина у здоровых лиц, с положительными и отрицательными результатами внутрикожных проб, в момент пробы был одинаков.

Авторы сравнивали результаты внутрикожных проб и прививочные реакции, выявляющиеся под действием адсорбированных тифозных вакцин, введенных подкожно 24 часа спустя. Тесной связи между ними не обнаружили, но определили, что после отрицательной внутрикожной пробы тяжелая местная прививочная реакция может ожидаться не более чем в 10% случаев, а также появление общей реакции является гораздо менее вероятным, чем после положительной внутрикожной пробы.

UNTERSUCHUNGEN ÜBER DIE NATUR DER TYPHUS-IMPFREAKTION

Von

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(Eingegangen am 15. August 1955)

In vorangegangenen Untersuchungen [1] wiesen wir mit Hilfe der Intrakutanprobe nach, dass dem mit Trichloressigsäure hergestellten Typhus-Extraktstoffe gegenüber gesunde Personen Überempfindlichkeit aufweisen. Unsere Beobachtungen deuten darauf hin, dass in den Impfreaktionen des hier gebräuchlichen, trichloressigsauren Extraktstoff enthaltenden RAUSSschen präzipitierten Typhus-Impfstoffs [2] auch allergische Faktoren eine Rolle spielen. Bei der Besprechung der Typhus-Impfreaktionen finden wir auch in der Literatur mehrere Hinweise auf die Bedeutung der individuellen Empfindlichkeit (RAUSS [3]; BARTOS [4]; RAETTIG, [5]).

Der trichloressigsaure Typhus-Bakterienextrakt wurde von DARVAS und UJHELYI [6] durch wiederholtes Ausfällen mit Alkohol gereinigt. Die an Mäusen festgestellte Toxizität des gereinigten Antigens konnte auf 1/10 des Ausgangswertes herabgesetzt werden, während der Immunisierungswert unverundert blieb. Der aus dem weniger toxischen Antigen bereitete Impfstoff verursachte jedoch unverändert starke Impfreaktionen [7, 8]. Auch diese Beobachtung lenkte die Aufmerksamkeit auf die Bedeutung der allergischen Komponente der Typhus-Impfreaktion. Die Allergeneigenschaft des Typhusantigens konnte von TOLNAI und HOLLÓS [9] in Tierversuchen unzweifelhaft nachgewiesen werden.

Die angeführten Literaturangaben und Beobachtungen veranlassten uns, die allergisierende Eigenschaft des präzipitierten Typhus-Impfstoffs nunmehr am Menschen unmittelbar zu untersuchen. Unserer Ansicht nach lässt sich die Frage klären, wenn wir die Impfreaktionen bei vorher mit Typhus-Impfstoff nicht geimpften Personen mit den Reaktionen mehrmals geimpfter Individuen vergleichen. Zeigen die vorher mehrfach geimpften Individuen schwerere Reaktionen, so ist die allergisierende Wirkung der Typhusimpfungen ohne Zweifel bewiesen.

Auf Grund dieser Überlegungen impften wir im März 1954 etwa 4000 (genau 4005) Personen gegen Typhus. Die Impfungen wurden in zwei voneinander entfernt liegenden Bezirken des Landes vorgenommen. Die Bevölkerung beider Bezirke bestand überwiegend aus Bauern. In einem der Bezirke war die gesamte Bevölkerung seit 1950 in jedem Jahr obligatorisch gegen Typhus geimpft

worden, während im anderen Bezirk Massenimpfungen gegen Typhus noch niemals durchgeführt wurden. In diesem Bezirk impften wir jedoch auch Personen, die aus irgendeiner Ursache (z. B. Militärdienst) vor 1950 schon einmal eine Schutzimpfung gegen Typhus erhalten hatten.

Die Impfungen wurden mit dem nach RAUSS hergestellten präzipitierten Typhus-Impfstoff (Typhylax, Fabr.-Nr. 13/18) vorgenommen. Die Dosis des präzipitierten Impfstoffs betrug 1 ml, doch erhielten Kinder unter 10 Jahren 0,5 ml ; die Impfung wurde an der Streckseite des linken Arms tief subkutan verabreicht.

Die Impfreaktionen vermochten wir in 3324 Fällen festzustellen. Von diesen hatten 1719 vorher überhaupt noch keine Schutzimpfung gegen Typhus erhalten 1605 dagegen waren schon vorher geimpft. Letztere wurden in zwei Gruppen eingeteilt. 797 Personen hatten vor 1950 eine, 808 Personen hingegen seit 1950 drei oder vier Typhus-Schutzimpfungen erhalten. Die angeführten Zahlen stellen in allen drei Gruppen 80—85% der geimpften Individuen dar.

Die Kontrolle der Reaktionen erfolgte am Tage nach der Impfung. Die Schutzimpfungen wurden von uns ausgeführt und auch die Reaktionen von uns selbst festgestellt. Die Untersuchung erstreckte sich auf den Umfang der an der Impfstelle auftretenden Infiltration und Hautrötung, auf die Schmerzhaftigkeit des Armes, Temperatur, Kopfschmerzen, allgemeine Schwäche, Erbrechen, Durchfall. Die Untersuchungsergebnisse gehen aus der Tabelle I hervor.

A) Infiltrationen. Aus der Tabelle ist ersichtlich, dass schwere lokale Impfreaktionen (Infiltrationen von 5 cm oder grösserem Durchmesser) bei den vorher mehrmals geimpften Personen etwa fünfmal häufiger vorkamen als bei vorher ungeimpften (vorher ungeimpfte Personen 5,9%, vorher einmal geimpfte 9,3%, vorher mehrmals geimpfte 27,8%).

B) Hautrötung. Zwischen den einzelnen Gruppen besteht kein bewertbarer Unterschied. Die Entwicklung der Hautrötung wird von den gegebenenfalls zur Anwendung gebrachten Umschlägen erheblich beeinflusst. Wahrscheinlich ist es darauf zurückzuführen, dass wir häufig verwaschene, landkartenartige, unregelmässig geformte Hautrötungen — wiederholt in grösserer Entfernung von der Impfstelle — sahen.

C) Schmerzen. Vorher geimpfte Personen wiesen ständige oder nur durch Bewegung ausgelöste Schmerzen in höherem Prozentsatz als vorher ungeimpfte auf. Bei der Bewertung dieser Differenz muss jedoch berücksichtigt werden, dass die Beurteilung der Schmerzen in hohem Masse auf subjektiver Empfindung beruht.

D) Fieber. Die Angaben über das Fieber zeigen, dass in dieser Hinsicht zwischen den einzelnen Gruppen kein Unterschied besteht. Es sei bemerkt, dass die Fieberreaktion meist innerhalb von 6—18 Stunden nach der Impfung abklingt und am folgenden Tage nur Subfebrilität beobachtet werden kann. Die Resultate können in hohem Masse durch Antipyretika beeinflusst werden.

Tabelle I

Impfreaktionen bei vorher mit Typhus nicht geimpften und geimpften Personen

Benennung	Vorher nicht	Vorher einmal	Vorher mehrmals	Vorher nicht	Vorher einmal	Vorher mehrmals
	Geimpfte			Geimpfte		
	in Zahlen			in Prozent		
Insgesamt	1719	797	808	100,0	100,0	100,0
	A) Infiltration					
∅	1364	605	190	79,3	75,9	23,5
1—4 cm	254	118	394	14,8	14,8	48,7
5 cm oder grösser	101	74	224	5,9	9,3	27,8
	B) Hautrötung					
∅	850	311	272	49,5	39,0	33,7
1—4 cm	297	133	243	17,3	16,7	30,1
5 cm oder grösser	572	353	293	33,2	44,3	36,2
	C) Schmerzen					
∅	981	190	179	57,1	23,8	22,1
Nur bei Bewegung	667	539	571	38,8	67,7	70,7
Ständig	71	68	58	4,1	8,5	7,2
	D) Fieber					
∅	1554	690	730	90,4	86,6	90,4
37,0—37,9°	112	84	59	6,5	10,5	7,3
38,0—38,9°	50	23	14	2,9	2,9	1,7
Über 39,0°	3	—	5	0,2	—	0,6
	E) Allgemeinsymptome					
Kopfschmerzen	284	299	151	16,5	37,5	18,7
Schwäche	103	101	60	5,9	12,7	7,4
Erbrechen, Durchfall	16	9	31	0,9	1,1	3,8

E) Allgemeinsymptome. Auch diese lassen sich wegen ihres subjektiven Charakters schwer bewerten. Am ehesten kann man hier noch die Angabe des Erbrechens und Durchfalls anerkennen. Erbrechen oder Durchfall kamen in der Gruppe der mehrmals Geimpften ungefähr viermal häufiger r als in den beiden anderen Gruppen.

Auf Grund dieser Beobachtungen können wir feststellen, dass zwischen den vorher mehrmals geimpften und vorhergehend nicht geimpften Personen ein entscheidender Unterschied in Erscheinung trat, und zwar gerade bei der am besten bewertbaren Reaktion, der lokalen Infiltration. In dieser Beziehung

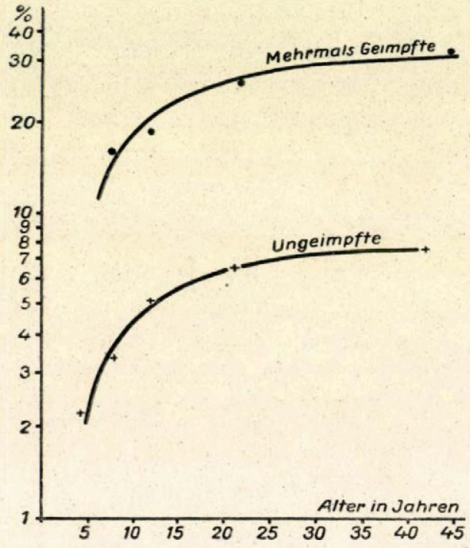

Abb. 1. Die Verteilung der schweren (5 cm oder grösseren Durchmesser aufweisenden) Infiltrationen nach Altersgruppen bei mehrmals Geimpften und Ungeimpften

fallen die Reaktionen der vorher einmal geimpften Individuen zwischen die der beiden anderen Gruppen.

Wir untersuchten auch die Verteilung der schweren (5 cm oder grösseren Durchmesser aufweisenden) Infiltrationen nach Altersgruppen (Abb. 1). Wie aus Abb. 1 ersichtlich, kamen schwere Impfreaktionen in sämtlichen Altersgruppen der mehrmals Geimpften in wesentlich höherem Prozentsatz vor als in den entsprechenden Altersgruppen der vorher gegen Typhus nicht Geimpften. Die Schwere der Impfreaktionen nahm jedoch mit dem Alter ausgeprägt zu, und zwar in den Gruppen der vorher nicht und der mehrmals Geimpften annähernd im gleichen Ausmass.

Besprechung

Die Untersuchungen ergaben, dass die gegen Typhus vorher mehrmals geimpften Personen auf die subkutane Einspritzung des präzipitierten Typhus-Impfstoffs mit schwererer Reaktion reagierten als die gegen Typhus vorher nicht geimpften. Aus dem Umstand, dass eine schwere Lokalreaktion (Infiltration von 5 cm oder grösserem Durchmesser) in der Gruppe der vorher mehrmals Geimpften annähernd in einem fünffach höheren Prozentsatz vorkam als unter den vorher Ungeimpften, geht deutlich hervor, dass die häufige Wiederholung der Typhusimpfungen zu wesentlich schwereren Reaktionen führt. Hieraus folgt, dass bei der Entwicklung der schweren Impfreaktionen der mehrmals Geimpften die gegenüber dem Typhusantigen entstandene Überempfindlichkeit eine wesentliche Rolle spielt. Jene Personen, die vor fünf oder mehr Jahren eine einzige Typhusimpfung erhalten hatten, zeigten nach unseren Angaben keine wesentlich schwerere Impfreaktion als die vorher noch nicht geimpften Individuen. Dies kann vielleicht darauf zurückgeführt werden, dass sich die Überempfindlichkeit nach einer Impfung noch nicht entwickelt, doch besteht auch die Möglichkeit, dass die Überempfindlichkeit auch nach einer einzigen Impfung zustande kommt, aber im Laufe von einigen Jahren verschwindet.

Mit der Beobachtung der Typhus-Impfreaktionen hat sich in Ungarn (im Zusammenhang mit dem präzipitierten Impfstoff) zuerst RAUSS [10] im Jahre 1937 befasst, als er über die Impfreaktionen bei 3400 Impflingen berichtete. Der damals benutzte Impfstoff war jedoch mit dem heutigen Typhylax nicht identisch, da er aus einem Antigen hergestellt wurde, dessen Extraktion durch Gefrieren und Wiedererwärmung erfolgte.

PÁTER [11] beobachtete 1944 die Typhusimpfreaktionen von 550 Personen, von denen jedoch nur 163 Typhylax erhalten hatten. Die Beobachtungen von ERDŐS [12] an etwa 700 Individuen erfolgten bereits in neuester Zeit. Diese beiden Autoren hatten ihre Untersuchungen zwecks Vergleich der Impfreaktionen verschiedener Typhus-Impfstoffe vorgenommen. In der ausländischen Literatur sind allergisch bedingte Hautveränderungen nach Typhusimpfung, wie Ekzem, Urtikaria, makulo-papulöses Exanthem, skarlatiniformes Exanthem, von mehreren Autoren beschrieben worden (FRIBOES, [13]; HECHT, [14]; NICOLAS und GATÉ, [15]; MOREL und GRUPPER, [16]; SOHIER, ALAIZE und PROUST, [17]). S. KIRCHMAYER [18] teilte einen Fall von hämorrhagischer Diathese nach Penta-Impfung mit.

Mehrere Autoren berichteten über die Entwicklung und Progression von neuralen Erkrankungen nach Typhus-Impfung (HIRSCH, 19; MOUSSAUD und WEISSENBACH, [20]; BANNWARTH, [21]; MUMME, [22], u. a.). Postvakzinöse neurale Komplikationen hält man heute im allgemeinen für allergisch bedingt (PETTE, [23]; KÖRNYEY, [24]; KORB, [25]). Laut WILSON und HADDEN [26], ferner PÁLFFY [27] können Nervenläsionen dadurch hervorgerufen werden,

dass sich auch in tiefer liegenden Geweben Ödem bzw. Urtikaria entwickelt, was zur Kompression der Nervenelemente führt. PÁLFFY sah nach Typhusimpfung Neuritis, Meningitis, Gesichts- und Augenmuskellähmung. Bei seinen Fällen trat fast ausnahmslos auch eine allergische Lokalreaktion auf. Eosinophilie entwickelte sich nach den Beobachtungen PÁLFFYS erst einige Wochen oder Monate nach der Impfung. LECOMTE und ROUSSELLE [28] beobachteten nach Typhus-Revakzination vorübergehendes Lungenödem. Demnach haben zahlreiche Autoren nach Typhus-Impfung verschiedene Impfkomplikationen beschrieben, die sie für allergisch bedingt hielten. Unsere Feststellungen deuten darüber hinausgehend darauf hin, dass auch die Lokalreaktion selbst allergischer Natur ist, da wir bestätigen konnten, dass die mehrmalige Wiederholung der Typhus-Impfungen zur Verschlimmerung der Impfreaktion führt. Man könnte demgegenüber einwenden, dass in einzelnen Fällen auch Personen, die vorher nicht gegen Typhus geimpft waren, auf die Impfung mit schwerer Reaktion reagierten. Dies lässt sich mit der toxischen Wirkung des Typhusantigens erklären, doch muss auch in Betracht gezogen werden, dass die Sensibilisierung des Organismus nicht nur parenteral erfolgen kann. Wir denken an die Rolle der latenten Infektionen, nach denen der Organismus sowohl dem Typhusantigen als auch anderen verwandten Antigenen gegenüber Sensibilität erwerben kann. Auf die Möglichkeit einer endogenen Allergisierung weist auch die Beobachtung hin, dass Typhus-Impfreaktionen mit zunehmendem Alter in schwererer Form auftreten (RAUSS [29]; RAETTIG [5] u. a.). Bei unseren eigenen Untersuchungen sahen wir das gleiche, und zwar sowohl bei den mehrmals als auch bei den vorher überhaupt noch nicht Geimpften. Der Unterschied zwischen diesen beiden Gruppen besteht lediglich darin, dass vorher nicht geimpfte Personen im Vergleich zu den mehrmals geimpften in allen Altersgruppen schwere Impfreaktionen in geringerem Prozentsatz aufwiesen. Dementsprechend laufen die auf Abb. 1 sichtbaren beiden Kurven in verschiedener Höhe, aber praktisch parallel miteinander. Der Umstand, dass wir die Verschlimmerung der Reaktionen mit zunehmendem Alter auch in der Gruppe der mit Typhus vorher nicht Geimpften nachzuweisen vermochten, weist auf die Rolle der endogenen Allergisierung hin. Es erscheint nicht unbedingt nötig, für das Zustandekommen der endogenen Allergisierung einen vorherigen Kontakt mit Typhusbakterien anzunehmen. In der Sensibilisierung kann auch anderen, heterologen Antigenen, insbesondere den Darmbakterien mit verwandter Antigenstruktur, eine Rolle zufallen. Diese Annahme wird auch durch die Versuche von SANARELLI [30] und von SDRODOWSKI [31] gestützt, die die Hypersensibilität von mit Cholera-Vibrionen, ja auch mit Eieiweiss vorbehandelten Tieren gegenüber Coli und anderen Bakterien nachzuweisen vermochten. Das SANARELLI—SDRODOWSKIsche Phänomen wurde von DARVAS, BORN und UJHELYI [32] auch bei der Erprobung des präzipitierten Cholera-Impfstoffs am Menschen beobachtet.

Zusammenfassung

1719 vorher mit Typhus nicht geimpfte, 797 vorher (vor 1950) mit Typhus einmal geimpfte sowie 808 vorher mit Typhus drei-viermal (nach 1950) geimpfte Personen erhielten zu gleicher Zeit eine Impfung mit präzipitiertem Typhus-Impfstoff. Die Feststellung der lokalen und Allgemeinreaktionen am folgenden Tage ergab, dass die mehrmalige Wiederholung der Typhus-Schutzimpfung dazu führt, dass die Impfreaktionen in wesentlich schwererer Form auftreten. In der Gruppe der mehrmals Geimpften wurden ungefähr fünffach schwerere Infiltrationen beobachtet als unter den vorher nicht geimpften Personen. Das prozentuale Verhältnis der schweren Infiltrationen stieg mit dem Alter, und zwar in gleicher Weise bei den mehrmals geimpften und vorher nicht geimpften Personen. Die Untersuchungsresultate ergaben, dass in der Entwicklung der Typhus-Impfreaktion die Überempfindlichkeit der geimpften Individuen eine wesentliche Rolle spielt.

*

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ИССЛЕДОВАНИЯ ХАРАКТЕРА ПРИВИВОЧНОЙ ТИФОЗНОЙ РЕАКЦИИ

ДЬ. ХЕДЬЕШИ, Ш. БОЖОКИ и К. УЛЬ

Резюме

Авторы производили противотифозную прививку адсорбированным прививочным материалом одновременно 1719 индивидов не вакцинированных предварительно тифом, 797 лиц привитых тифом однократно (до 1950 года), а также 808 лиц предварительно трёх-четырёхкратно привитых тифом (после 1950 года). Сочтя на следующий день местные и общие реакции, авторы определили, что повторяемые несколько раз противотифозные прививки приводят к отяжелению прививочных реакций. В неоднократно привитой группе наблюдалось приблизительно в пять раз больше тяжелых инфильтратов, чем среди непривитых предварительно лиц. Процентное отношение тяжелых инфильтратов повышалась с возрастом как в группе неоднократно привитых, так и в группе предварительно не вакционированных лиц. В результате исследований авторы определили, что в развитии тифозных прививочных реакций значительную роль играет повышенная чувствительность привитых лиц.

ÜBER DIE SCHWIERIGKEITEN DER TYPENBESTIMMUNG INFOLGE DES EINFLUSSES DES INH AUF DAS M. TUBERCULOSIS

Von

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Die humane Variante des *M. tuberculosis* wurde bisher von der bovinen folgendermassen unterschieden:

1. auf Grund der morphologischen Eigenschaften der Kolonien,
2. auf Grund der auf das Kaninchen bezogenen Virulenz.

1. Auf Grund der morphologischen Eigenschaften der Kolonien erfolgt die Auswahl der Stämme *(Jensen)*. Zu den Eigenschaften eines humanen Stammes gehören:
a) Glyzerin fördert sein Wachstum (Eugonie),
b) er ist von gelber Farbe,
c) die Oberfläche der Kolonie ist rauh (rough),
d) die Kolonie ist trocken, bröckelig.
Zu den Eigenschaften eines bovinen Stammes gehören:
a) Glyzerin beeinflusst über 0,5% das Wachstum ungünstig (Dysgonie),
b) er ist farblos,
c) die Oberfläche der Kolonie ist glatt (smooth),
d) die Kolonie ist leichter ausstreichbar und weniger bröckelig als der humane Typus.
2. 0,01 mg des bovinen Typus verursacht beim Kaninchen 2 Monate nach intravenöser Impfung eine käsige Pneumonie.
Der humane Typ ruft entweder gar nichts hervor, oder nadelstichgrosse schiefergraue regressive Veränderungen in der Lunge. Auf Grund der obigen Eigenschaften können folgende Typen vorkommen:
a) eugonisch, mit verminderter Kaninchenvirulenz (human),
b) dysgonisch, mit gesteigerter Kaninchenvirulenz (bovin),
c) eugonisch, mit gesteigerter Virulenz,
d) dysgonisch, mit verminderter Virulenz } Übergangstypen.
Das Vorkommen von reinen Typen im menschlichen Sektionsmaterial ist selten, worauf wir bei unseren im Jahre 1950 an einem grossen Material durchgeführten Untersuchungen ebenfalls hingewiesen haben [1]. Die Umwandlung der Stämme wurde von FRIMODT-MÖLLER [2], JENSEN [3], WAGENER und MITSCHERLICH [4] beobachtet. WEISSFEILER [5] wies darauf hin, dass die Umwandlung aus der Kolonienform S in die Kolonienform R von konstantem Charakter ist und ihre Schnelligkeit auch von den gegebenen Umständen beeinflusst werden kann. Da im menschlichen pathologischen Material die Zahl der reinen Typen der Mycobakterien selten ist, ist es offensichtlich, dass der grössere Teil der Stämme an irgendeinem Punkt der S—R »Skala« seinen Platz einnimmt. Die Einreihung der Übergangstypen unter die humanen oder bovinen Typen beansprucht eine grosse Umsicht und Fachkenntnis.

Die Einführung des INH in die Therapie bedeutete neue Umstände für das Bakterium, die viele bekannte Eigenschaften des Krankheitserregers veränderten.

Im Laufe unserer diagnostischen Routinearbeit in den letzten 2 Jahren beobachteten wir ebenfalls das Phänomen der unter dem Einfluss des INH

auftretenden Hormesis, die schnell steigende in vitro Resistenz, die langsame Wachstumsfertigkeit der resistenten Stämme auf dem Nährboden sowie die verminderte Virulenz dieser Stämme [6]. Während der Typenbestimmungsarbeit ist uns aufgefallen, dass aus alten und bekanntlich mit R-Typ (human) infizierten Kranken zur Zeit der INH-Behandlung dem S-Typ ähnliche, blasse, kaum pigmentierte Stämme mit glatter Oberfläche in grosser Anzahl gezüchtet werden konnten.

Diese morphologische Veränderung der Stämme brachten wir mit der Wirkung des INH in Zusammenhang. Auch in der Fachliteratur fanden wir diesbezüglich Angaben [7]. Die erwähnte morphologische Veränderung verursachte uns bei der Typenbestimmungsarbeit vom Standpunkt der Absonderung Schwierigkeiten. Um den Einfluss des INH auf eine diesbezügliche Veränderung der Stämme beobachten zu können, nahmen wir Untersuchungen bzw. Versuche vor.

Experimenteller Teil

1. Im ersten Teil unserer Arbeit wiederholten wir mit dem im Jahre 1955 im Laboratorium eingetroffenen Untersuchungsmaterial die Typenbestimmungsarbeit, die von einem der Verfasser in den Jahren 1950/51 durchgeführt wurde. Unser Ziel war, im Spiegel der obenerwähnten Beobachtungen und der INH-Wirkung das mehrere Zehntausend Fälle betragende Material unseres Laboratoriums zu überprüfen, das im wesentlichen die M. tuberculosis-Population des ganzen Landes vertritt und so vom Standpunkt der Typenbestimmung eventuell neue epidemiologische Angaben zu liefern vermag.

Das vollständige diagnostische Routinematerial — den Kehlkopftampon ausgenommen — wurde auf 2 LÖWENSTEIN-JENSENsche feste und 2 ŠULAsche flüssige Nährböden geimpft. Von den 2 festen Nährböden enthielt der eine 0,25%, der andere 0,75% Glyzerin. Die flüssigen Nährböden wurden mit denselben Glyzerinkonzentrationen bereitet. Die Untersuchungsstoffe wurden nach einer in unserer Abteilung ausgearbeiteten, allgemein verwendeten und gut bewährten Vorbehandlung mit 10%igem Triphosphat geimpft [10]. Die beimpften Nährböden wurden nach Inkubation von 4, 6, 8 Wochen bei 37°C abgelesen bzw. typisiert. Wo wenige Kolonien erschienen oder wo wegen einer anderen Ursache eine Wiederholung vorgenommen werden musste, wurden nach entsprechender Homogenisierung ebenfalls auf den obigen 4 Nährböden Subkulturen bereitet.

Auf Grund der morphologischen Eigenschaften der Kolonien wurden 99 Stämme ausgewählt, die für bovinen Typus gehalten wurden. Ihre INH-Empfindlichkeit in vitro wurde festgestellt. Die Bestimmung erfolgte im ŠULAschen Nährboden mit verschiedenem INH-Gehalt bei 1, 2, 4, 8, 16 γ/ml pro Röhre sowie in 2 Kontrollröhren. Das Inokulum betrug 1,0 mg, die Inkubationszeit 3 Wochen.

Die Ergebnisse sind in Tabelle I veranschaulicht. Von den 99 für bovinen Typ erachtbaren Stämmen erwiesen sich 86,9% als INH-resistent.

Diese Angabe, wenn wir berücksichtigen, dass 87,9% der Stämme aus pulmonalem Material stammte, rechtfertigt bereits allein unsere Annahme, dass sich die morphologischen Eigenschaften der Kolonien humanen Typs unter dem Einfluss des INH verändern und dem bovinen Typ ähnlich werden.

Tabelle I

INH-Empfindlichkeit der untersuchten Stämme

Herkunft der Stämme	Resistenz γ/ml					Empfindlich		Zusammen
	1	2	4	8	16	Zahl	%	
Pulmonal	21	17	11	6	21	12	13,6	88
Extrapulmonal ...	2	7	—	1	—	1	9,1	11
Zusammen	23	24	11	7	21	13	13,1	99

Wenn wir auch voraussetzen, dass die Mehrzahl der Stämme aus extrapulmonalem Material zum bovinen Typ gehörte, kann festgestellt werden,

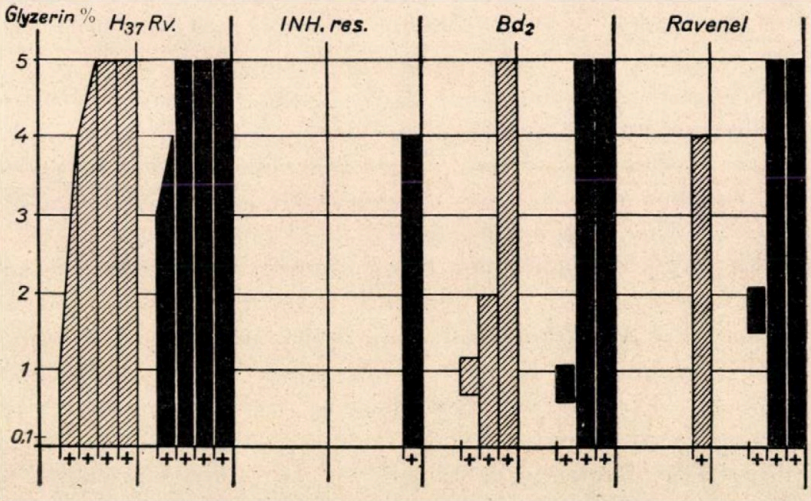

Abb. 1. Wachstum von M. tuberculosis-Stämmen auf LÖWENSTEINschem Nährboden bei Veränderung der Glyzerinkonzentration (Inokulum : 10^{-5} mg) *Anmerkung:* Die Zahl der Kreuze bezeichnet die Grösse der Kolonien

dass die INH-Resistenz in den echten bovinen Stämmen zumindest in demselben Verhältnis vorkommt wie in den humanen Stämmen.

2. In unserem zweiten Versuch wurde die Wirkung der verschiedenen Glyzerinkonzentrationen auf die Morphologie der Kolonien an den in unserem Laboratorium erhaltenen Standardstämmen untersucht.

Wir untersuchten 4 Stämme :

a) Ravenel (ein alter internationaler boviner Laboratoriumsstamm),

b) Bd_2 (ein von uns seit 4 Jahren aus menschlicher Lymphknotentuberkulose isolierter, identifizierter und erhaltener boviner Stamm),

c) $H_{37}Rv$,

d) von CZANIK und LEHOCZKY gegenüber INH von 100 γ/ml Konzentration resistent gemachte Variante des $H_{37}Rv$.

Wir impften die Stämme in einer Verdünnung von 10^{-4}—10^{-8} mg/ml auf LÖWENSTEIN—JENSENsche und ŠULAsche Nährböden mit 0,1, 0,3, 0,5, 0,8, 1,0, 2,0, 4,0, 5,0% Glyzeringehalt. Das Mass des Wachstums und die morphologischen Eigenschaften wurden am 14. und 28. Tage abgelesen.

Die Ergebnisse sind auf Abb. 1 veranschaulicht, auf der die prozentuelle Glyzerinkonzentration des Nährbodens dem Mass des Wachstums, genauer der Grösse der Kolonien gegenübergestellt wurde. (Dies wurde mit Kreuzen bezeichnet.)

Wir konnten feststellen, dass die zwei Bovinstämme sowohl am 14. als auch am 28. Tage ein langsames und im allgemeinen schwaches Wachstum, zeigten. Der verhältnismässig niedrige Glyzeringehalt förderte ihr Wachstum, der optimale Wert der Glyzerinkonzentration war zwischen 0,5 und 0,8%.

Der $H_{37}Rv$-Stamm wuchs bei einem Glyzeringehalt von 0,1—4% gut, während 5% Glyzerin das Wachstum bereits hemmte.

Die INH-resistente Variante des $H_{37}Rv$ wuchs, von der Glyzerinkonzentration vollkommen unabhängig, in zwei Wochen überhaupt nicht und auch in der vierten Woche nur schwach. Unter den obigen Bedingungen ist der Unterschied zwischen den einzelnen Stämmen hinsichtlich der Wachstumsfähigkeit am 14. Tag ausgesprochener.

Die Abweichung bezüglich der Pigmentierung der Stämme ist am 14. Tag nicht auffallend. Am 28. Tag sind die Bovinstämme blass und weisen eine kaum merkbare Verfärbung auf. Das $H_{37}Rv$ ist gelb pigmentiert. Die INH-resistente Variante des $H_{37}Rv$ ist vollkommen farblos, durchscheinend.

Obwohl es schwierig ist, die Oberflächenunterschiede der Kolonien pünktlich zu beschreiben, stellten wir bei der Ablesung nach 4 Wochen folgendes fest. Die Oberfläche der bovinen Stämme ist glatt, ihre Einziehungen sind oberflächlicher, die Oberfläche der einzelnen begrenzten Teile weniger rauh. Das ganze Bild ähnelt dem Blumenkohl. Das $H_{37}Rv$ zeigt die typische Kolonienform R : tiefe Einziehungen an der Oberfläche, die von den Einziehungen begrenzten Teile haben eine viel rauhere Oberfläche als die Einziehungen der Kolonien der bovinen Stämme. Das Bild ist der Himbeere ähnlich. Die Oberfläche der INH-resistenten Variante des $H_{37}Rv$ zeigt eher eine Kolonienform S. Obwohl an der Oberfläche der Kolonien die tiefen Einziehungen anzutreffen sind, weisen doch die durch sie begrenzten Teile eine sehr feine, fast samtene Oberfläche auf. Das Gesamtbild der Kolonien ähnelt der Oberfläche des Griessbreis. Im ganzen ist die *glatte, durchscheinende Farblosigkeit* der Kolonien als charakteristisch zu bezeichnen.

Die Oberflächeneigenschaften sind also — besonders in jüngeren Kulturen — zwischen den INH-resistenten und den bovinen Stämmen sehr verwaschen.

Auf Abb. 2 stellen wir eine Aufnahme von den obigen vier Stämmen auf LÖWENSTEIN—JENSENschem Nährboden mit 4%igem Glyzeringehalt im Alter von 4 Wochen bei einem Inokulum von 10^{-5} mg/ml dar. Der Farbenunterschied zwischen dem $H_{37}Rv$ und dem bovinen Stamm Bd_2 ist gut wahrnehmbar. Der Oberflächenunterschied zwischen den Stämmen bzw. die auffallende Ähnlichkeit zwischen dem bovinen und dem resistenten Stamm können beobachtet werden. Die Dys- bzw. Eugonie im Zusammenhang mit dem Wachstum der Stämme sind ebenfalls sichtbar.

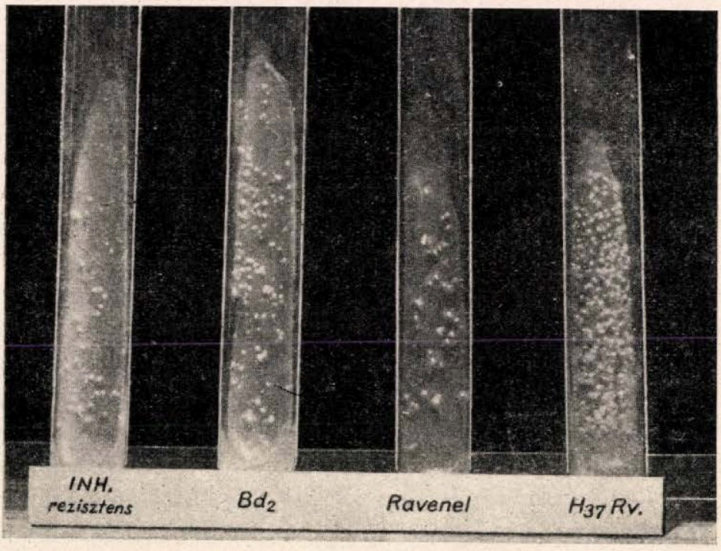

Abb. 2. Wachstum von verschiedenen Stämmen auf LÖWENSTEIN—JENSENschem Nährboden nach 28tägiger Inkubation (Inokulum: 10^{-5} mg/ml)

Besprechung

An Hand der Untersuchungen konnten wir feststellen, dass die Absonderung der Typen der aus menschlichem pathologischem Material gezüchteten M. tuberculosis-Stämme auf Grund der bisher bekannten und beschriebenen morphologischen Eigenschaften der Kolonien noch mehr erschwert ist, weil die INH-resistenten Stämme dem (bovinen) Typ S ähnliche Eigenschaften aufnehmen. Die Stämme also, die INH gegenüber ihre Empfindlichkeit verloren haben bzw. resistent geworden sind, verändern sich auch in ihren morphologischen Eigenschaften. Der in den veränderten morphologischen Eigenschaften der Kolonien zum Ausdruck gelangende Wechsel bleibt in weiteren Subkulturen ebenfalls erhalten. Im Zusammenhang mit unseren Versuchen taucht die Frage auf, ob der Einfluss der Glyzerinkonzentration des Nährbodens auf das Wachstum, das bisher bei der Identifizierung des Typs der

Stämme für eine sehr charakteristische Eigenschaft gehalten wurde, auch weiterhin für die Typisierung verwendbar ist.

Wir fanden, dass der Glyzeringehalt das Wachstum der INH-resistenten Stämme selbst in Primokulturen nicht beeinflusst. Da DREA, sich auf die Arbeiten von GRIFFITH, EVANHOFF und SWEANY [8] berufend, feststellt, dass auch zahlreiche reine bovine Stämme in Anwesenheit von Glyzerin gut wachsen, können wir behaupten, dass diese Eigenschaft vollkommen wertlos wird und als eine charakteristische Eigenart bei der Absonderung nicht zu verwenden ist.

Noch eine Frage kann gestellt werden, nämlich, ob die bovinen Stämme INH gegenüber eventuell schneller resistent werden als die humanen. FUST und BÖHNI [9] stellten fest, dass auch die bovinen Stämme nicht eher resistent werden als die humanen.

An Hand unserer Beobachtungen können wir also feststellen, dass die Auswahl der aus menschlichem pathologischem Material stammenden M. tuberculosis-Stämme auf Grund der morphologischen Eigenschaften der Kolonien auf LÖWENSTEINschem Nährboden mit 0,25- und 0,75%igem Glyzeringehalt unter dem Einfluss des INH in grosser Anzahl einen dem bovinen Typ ähnlichen Stamm ergab, der weder ein boviner Stamm noch einer von intermediärem Typ ist. Diese neue Eigenschaft der Stämme bildet sich unseres Erachtens bereits bei einer INH-Resistenz von 1—2 γ heraus, und die Stämme bewahren diese neue Eigenart auch in Subkulturen.

In Zukunft müssen wir also, wenn wir die Typenbestimmung richtig durchführen wollen, auch die INH-Resistenz bzw. -Empfindlichkeit der auf Grund der morphologischen Eigenschaften der Kolonien ausgewählten Stämme untersuchen. Die sich so herausgebildeten resistenten, ihre Empfindlichkeit verlorenen Stämme können mit den bisherigen Methoden nicht mehr typisiert werden, weil ihre Virulenz in bezug auf Meerschweinchen und Kaninchen abgenommen hat. Daraus folgt, dass uns die Stämme von bestimmt bovinem Ursprung verloren gehen, wenn sich die gezüchteten Stämme als INH-resistent erweisen.

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ТРУДНОСТИ ОПРЕДЕЛЕНИЯ ТИПОВ ВСЛЕДСТВИЕ ДЕЙСТВИЯ INH НА M. TUBERCULOSIS

И. САБО и Л. ЛУГОШИ

Резюме

Авторы из давнейших случаев туберкулёза легких известно человеческого типа в процессе лечения INH выделили дисгонические штаммы обладающие морфологическими особенностями присущими колониям бычьего типа. 86,9% этих штаммов были резистентны к INH.

Ввиду того, что штаммы сохраняют эти свойства, обладают пониженной вирулентностью для морских свинок и кроликов, авторы пришли к выводу, что в процессе работ по классификации типов нужно определить резистентность к INH. Типы резистентных штаммов не могут быть определены.

NERVENSYSTEM UND IMMUNITÄT VI.

DIE WIRKUNG VON DAUERSCHLAF AUF DAS ARTHUS-SACHAROW-PHÄNOMEN

Von

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Laut unserer früheren Versuche [1] verzögert bzw. hemmt der durch die subkortikal wirkende Phenyläthylbarbitursäure hervorgerufene Dauerschlaf die Anreicherung der Immunkörper im Blute. Im Zusammenhang mit dieser Feststellung wurde auch darauf hingewiesen, dass der Dauerschlaf auf zweierlei Weise einen im Vergleich zu den Kontrollen niedrigeren Immunkörpertiter des Blutes verursachen kann: entweder dadurch, dass er einfach die Bildung von Immunkörpern hemmt; oder aber dadurch, dass zwar die Immunkörperbildung während des Dauerschlafes mit physiologischer Intensität und Geschwindigkeit stattfindet, dass aber die Gewebezellen der mit Phenyläthylbarbitursäure narkotisierten Tiere mehr in physiologischer Menge gebildete Immunglobuline adsorbieren bzw. binden, so dass auf diese Weise weniger Immunkörper in die Blutbahn gelangen.

Diese für die Theorie wie für die Praxis gleicherweise wesentliche Frage lässt sich nur durch die Bestimmung der an die Gewebe adsorbierten Immunkörpermenge in exakter Weise klären. Zur Bestimmung der an die Gewebe gebundenen Immunkörper steht aber vorläufig keine erprobte Methode zur Verfügung, so dass wir gezwungen waren, Versuche zur Ausarbeitung einer neuen diesbezüglichen Methode in Angriff zu nehmen. Vorher bzw. gleichzeitig wurde versucht, auf Grund der lokalen Anaphylaxie, des ARTHUS—SACHAROW-Phänomens, Einblick in den Mechanismus zu erhalten, durch den der Dauerschlaf die Menge der im Blut nicht zirkulierenden, an die Gewebe adsorbierten Immunkörper beeinflusst. Wie bekannt, beruht das ARTHUS—SACHAROW-Phänomen auf den an die Zellen gebundenen, adsorbierten Immunkörpern, so dass nach unseren Vorstellungen, wenn sich die Immunkörperbildung während des Dauerschlafes nicht ändert und nur in der Verteilung der gebildeten Immunkörper eine Verschiebung zu Lasten der in der Blutbahn zirkulierenden Immunkörper und zu Gunsten der adsorbierten Immunkörper eintritt, die lokale Anaphylaxie an den narkotisierten Tieren in kürzerer Zeit und intensiver in Erscheinung treten muss.

Methodik und Versuchsergebnisse

Als Versuchstiere wurden Kaninchen verwendet. Allen denen, die über Erfahrungen in Verbindung mit der Entstehung und Auswertung der lokalen Anaphylaxie verfügen, ist es wohlbekannt, dass zwischen den einzelnen Kaninchen — selbst bei gleichen Ernährungs- und Haltungsverhältnissen — grosse individuelle Unterschiede hinsichtlich der zur Auslösung des ARTHUS—SACHAROW-Phänomens notwendigen Zeit und Antigenmenge bestehen und dass infolge dieser grossen individuellen Streuung selbst an einer sehr grossen Anzahl von Tieren ausgeführte Versuche nicht immer ein leicht auswertbares Ergebnis zeitigen. Gerade aus diesem Grunde wurde zur Ausschaltung der individuellen Unterschiede bei unseren Versuchen die Selbstkontrolltechnik gewählt. An jedem Tier wurden die zur Hervorrufung des ARTHUS—SACHAROW-Phänomens notwendige Antigenmenge, die Zahl der Injektionen, der Zeitpunkt des Erscheinens der Reaktion und die Grösse des nekrotischen Gebietes sowohl in der Narkose als auch in wachem Zustand bestimmt. Die im Dauerschlaf ermittelten Werte wurden stets mit den in wachem Zustand desselben Tieres festgestellten Werten verglichen.

Zur Auslösung des ARTHUS—SACHAROW-Phänomens wurde die Methode von GERMUTH und OTTINGER [2] angewandt: die Versuchstiere erhielten täglich intrakutan je 0,1 ml steriles Pferde- bzw. Rinderserum in den depilierten Hautbezirk auf dem Rücken. Es wurde normales Pferdeserum der Staatlichen Impfstoffproduktionsanstalt «Phylaxia» (Fabr. Nr. 51/1; Stamm Nr. 70 024) bzw. vom Schlachthof in Debrecen stammendes steriles Rinderserum verwendet. Hinsichtlich der Stärke des ARTHUS—SACHAROW-Phänomens haben diese beiden Antigene früheren Erfahrungen zufolge annähernd dieselbe Wirkung, eine Tatsache, die auch durch die neueren Versuche bestätigt wurde.

Tabelle I

Kaninchen		Kontrollkaninchen						Abweichung in der	
		Rechte Seite 0,1 ml Rinderserum i. c.			Linke Seite 0,1 ml Pferdeserum i. c.				
Nr.	Gewicht kg	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zeit Tage	Antigenmenge ml
171.	3,1	6	7	0,3	7	9	0,3	+ 2	0,1
172.	3,0	8	10	0,3	8	10	0,1	—	—
173.	3,0	9	11	0,15	10	12	0,3	+ 1	0,1
174.	3,1	9	11	0,2	11	13	0,7	+ 2	0,2

Die obigen 4 Tiere erhielten täglich zur gleichen Zeit in wachem Zustand in zwei symmetrische Stellen der depilierten Rückenhaut intrakutan je 0,1 ml Pferde- bzw. Rinderserum. Wie aus Tabelle I ersichtlich, löste das Rinderserum einen etwas stärkeren Effekt aus, indem es das ARTHUS—SACHAROW-Phänomen um 1 bis 2 Tage früher bzw. mit einer um 0,1 bis 0,2 ml geringeren Serummenge hervorrief. Dieser Unterschied wurde in den Hauptversuchen jedenfalls im Laufe der Auswertung stets in Betracht gezogen. Aus der Tabelle geht ferner hervor, dass die Messung der Grösse des nekrotischen Gebietes keine geeignete Vergleichsgrundlage darstellt, da die Intensität der Reaktion selbst an ein und demselben Kaninchen stark unterschiedlich sein kann.

Zu den weiteren Versuchen wurden 20 Kaninchen herangezogen. Die Hälfte der Tiere erhielt jeden zweiten Tag intraperitoneal 10 mg Phenyläthylbarbitursäure je 100 g Körpergewicht, was etwa 6 bis 14 Stunden tiefen Schlaf und danach 12 bis 16 Stunden Schlummern zur Folge hatte. In der Zwischenzeit ernährten sich die Tiere spontan, so dass diese jeden zweiten Tag ausgeführte Narkose das Körpergewicht im Vergleich zu den nicht narkotisierten Kontrolltieren nicht wesentlich beeinflusste. Während dieser Zeit wurde den Tieren täglich 0,1 ml Pferdeserum intrakutan verabreicht. Beim Auftreten des ARTHUS—SACHAROW-Phänomens wurde die Narkosebehandlung unterbrochen, 7 bis 15 Tage erhielten die Tiere keinerlei Behandlung, nach dieser Ruheperiode wurde dann täglich ohne Narkose 0,1 ml Rinderserum in das symmetrische, depilierte Gebiet der Rückenhaut intrakutan eingespritzt. Die anderen 10 Tiere erhielten zuerst täglich in wachem Zustand Rinderserum, ruhten dann 10 bis 16 Tage nach dem Auftreten des ARTHUS—SACHAROW-Phänomens, wurden nach dieser Ruheperiode jeden zweiten Tag mit Sevenal eingeschläfert und täglich mit Pferdeserum behandelt. Die diesbezüglichen Ergebnisse sind in Tabelle II zusammengestellt.

Von den 20 Tieren gingen 2 an interkurrenten Krankheiten ein, u. zw. eines während der Narkose und eines in wachem Zustand. Unter den übriggebliebenen 18 Tieren beeinflusste der Dauerschlaf bei 8 Kaninchen die Entstehung des ARTHUS—SACHAROW-Phänomens nur in einer wertmässig nicht erfassbaren Weise. Bei 10 Tieren dagegen hemmte die Narkose das durch Pferdeserum ausgelöste ARTHUS—SACHAROW-Phänomen ausgesprochen im Vergleich zu der in wachem Zustand durch Rinderserum hervorgerufenen lokalen Anaphylaxie. Bei 5 Kaninchen war 0,3 bis 0,8 ml mehr Serum während der Narkose notwendig, um eine im Vergleich zu dem in wachem Zustand verabreichten Rinderserum 4- bis 10tägige Verzögerung des Auftretens des ARTHUS—SACHAROW-Phänomens zu verursachen. An weiteren 5 Tieren konnte durch täglich in der Narkose gegebene 26 bis 28 Pferdeseruminjektionen selbst während eines Monats nicht das ARTHUS—SACHAROW-Phänomen hervorgerufen werden, obwohl dieselben Kaninchen vorher oder nachher nach 4 bis 12 Injektionen eines ein ungefähr gleiches Sensibilisierungsvermögen besitzenden

Tabelle II

Kaninchen		0,1 ml Pferdeserum i. c. in Narkose			0,1 ml Rinderserum i. c. in wachem Zustand			Abweichung von wachem Zustand	
Nr.	Gewicht kg	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zeit Tage	Antigenmenge ml
175.	2,5	28	31	0	5	7	0,3	24	2,3
176.	3,8	28	31	0	8	10	0,7	21	2,0
177.	3,3	26	30	0	4	6	0,4	24	2,2
178.	3,4	26	30	0	5	7	0,2	23	2,1
179.	2,8	28	31	0	12	15	0,4	16	1,6
180.	2,7	16	20	0,2	13	15	0,6	5	0,3
181.	3,0	15	19	0,2	7	9	0,4	10	0,8
182.	2,9	15	18	0,2	10	13	0,4	5	0,15
183.	3,5	14	17	0,6	7	9	0,6	8	0,7
184.	3,5	9	11	0,5	6	7	0,2	4	0,3

Bei den Kaninchen Nr. 185—192 wurde das ARTHUS—SACHAROW-Phänomen durch die Narkose nicht beeinflusst.

Rinderserums in den üblichen 6 bis 15 Tagen — ähnlich wie in den Kontrollversuchen — mit einer klassischen ARTHUS—SACHAROW-Phänomen reagierten. Im Endergebnis lässt sich also feststellen, dass die von uns angewandte Narkosebehandlung an der Hälfte der Tiere das durch Pferdeserum auslösbare ARTHUS—SACHAROW-Phänomen ausgesprochen hemmte bzw. verzögerte.

In der folgenden Versuchsreihe mit 20 Tieren sollte in umgekehrter Anordnung die lokale Anaphylaxie durch Verabreichung von Rinderserum während der Narkose mit vorheriger bzw. nachheriger Gabe von Pferdeserum in wachem Zustand ausgelöst werden.

Wie aus Tabelle III hervorgeht, beeinflusste bei 9 von 20 Tieren die Narkose das ARTHUS—SACHAROW-Phänomen nicht in auswertbarer Weise. Bei 11 Tieren war dagegen während des Dauerschlafes um 0,3 bis 1,8 ml mehr Rinderserum notwendig, um eine 3- bis 19tägige Verzögerung des Auftretens des ARTHUS—SACHAROW-Phänomens im Vergleich zu der in wachem Zustand erfolgenden Pferdeserumbehandlung zu verursachen.

Nach den bisherigen Ergebnissen muss man also ebenfalls zu der Folgerung gelangen, dass der Dauerschlaf bei etwa der Hälfte der Tiere auch das durch Rinderserum hervorgerufene ARTHUS—SACHAROW-Phänomen verzögert.

Tabelle III

Kaninchen		0,1 ml Rinderserum i. c. in Narkose			0,1 ml Pferdeserum i. c. in wachem Zustand			Abweichung von wachem Zustand	
Nr.	Gewicht kg	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zahl der Injektionen	Auftreten der Nekrose in Tagen	Fläche der Nekrose in cm²	Zeit Tage	Antigenmenge ml
193.	3,2	23	26	0,4	5	7	0,5	19	1,8
194.	3,5	17	21	0,2	11	14	0,6	7	0,6
195.	4,0	11	13	0,2	8	10	0,2	3	0,3
196.	3,2	10	12	0,3	6	8	0,5	4	0,4
197.	3,8	19	22	0,5	8	10	0,3	12	0,9
198.	3,8	25	28	0,3	10	13	0,2	15	1,5
199.	3,5	14	16	0,2	5	7	0,4	9	0,9
200.	3,4	23	26	0,4	9	11	0,2	15	1,4
201.	3,7	14	17	0,3	6	8	0,7	9	0,8
202.	3,0	15	17	0,2	7	9	0,3	8	0,8
203.	3,2	20	22	0,5	7	9	0,3	13	1,3

Bei den Kaninchen Nr. 204—212 wurde das ARTHUS—SACHAROW-Phänomen durch die Narkose nicht beeinflusst.

Diskussion

Die hier beschriebenen Versuche zeigen also, dass das ARTHUS—SACHAROW-Phänomen infolge der Narkose bei etwa der Hälfte der Versuchstiere verspätet auftritt und in einzelnen Fällen sogar vollständig ausbleiben kann. Bei der Besprechung der Ergebnisse ist vor allem darauf hinzuweisen, dass die Wirkung der Narkose keineswegs so eindeutig ist, wie dies im Zusammenhang mit dem Erscheinen der Immunkörper im Blut zu sehen war, wo bei jedem Versuchstier infolge der Narkosewirkung immer eine Verzögerung des Erscheinens der Immunkörper beobachtet werden konnte. Diese Abweichung zwischen den zwei Versuchsreihen zeigt also auch weiterhin, dass es unumgänglich notwendig ist, die Menge der an die Gewebe gebundenen Immunkörper zu bestimmen, um den quantitativen Ablauf der Immunkörperproduktion exakt beurteilen zu können.

Es eröffnet sich natürlich ein weites Gebiet für Spekulationen, wenn man begründen wollte, warum der Dauerschlaf bei der Hälfte der Versuchstiere keine Wirkung auf die Ausbildung des ARTHUS—SACHAROW-Phänomens ausübte. Man könnte daran denken, dass die angewandte Narkose bei diesen Tieren nicht genügend stark zur Hemmung des ARTHUS—SACHAROW-Phäno-

mens war und dass drastischere Dosen bei häufigerer Narkotisierung auch in diesen negativen Fällen die Hemmung ausgelöst hätten. Es besteht ferner die Möglichkeit, dass die unterschiedlichen Nervensystemtypen der Kaninchen eine Erklärung für die uneinheitlichen Versuchsergebnisse zu bieten vermögen. Zieht man nun in Betracht, dass dieselbe Narkosebehandlung in jedem einzelnen Fall ausnahmslos die Menge der im Blute zirkulierenden Agglutinine vermindert hatte, während diese Verminderung der an die Gewebe adsorbierten Immunkörper nur in der Hälfte der Fälle eintrat, so wird man den Schluss ziehen, dass der Dauerschlaf nicht nur die Bildung der Immunglobuline beeinflusst, sondern auch auf deren Verteilung zwischen Blut und Geweben eine Wirkung auszuüben vermag. Die Untersuchung all dieser hier aufgeworfenen Möglichkeiten stellt die Aufgabe weiterer Versuche dar.

Demgegenüber ist unbedingt als feststehend hervorzuheben, dass der Dauerschlaf bei unseren Versuchstieren entweder wirkungslos war oder in der anderen Hälfte der Fälle das Auftreten der lokalen Anaphylaxie, die auf den an die Gewebe adsorbierten Antikörpern beruht, ausgesprochen ebenso hemmte, wie dies bereits in Verbindung mit den in der Blutbahn zirkulierenden Immunkörpern festgestellt wurde. Hieraus ist der Schluss zu ziehen, dass während des Dauerschlafes auch die Immunkörperproduktion selber gehemmt ist und dass es sich nicht um den Fall handelt, wo die Immunkörperproduktion in den narkotisierten Tieren mit einer physiologischen Intensität und Geschwindigkeit stattfindet, wobei die Gewebe der narkotisierten Tiere mehr Immunglobuline binden und weniger in die Blutbahn gelangen lassen. Dieser Möglichkeit würde nämlich jenes Ergebnis entsprechen, bei dem das ARTHUS—SACHAROW-Phänomen während der Narkose rascher und intensiver auftritt. Dies konnte jedoch nicht festgestellt werden.

Schliesslich muss noch der folgende Einwand erörtert werden. Es ist zweifellos, dass der Pathomechanismus des ARTHUS—SACHAROW-Phänomens überaus kompliziert ist, so dass es schwer fällt, die einfachen Zusammenhänge klar nachzuweisen. Das Ausbleiben und die Verzögerung der Reaktion lässt sich nämlich auch dadurch erklären, dass die Reaktionsbereitschaft der Haut im Dauerschlaf abnimmt, ferner auch dadurch, dass das Zusammentreffen des Antigens mit den an die Gewebe gebundenen Antikörpern bzw. der Ablauf der Antigen-Antikörper-Reaktion infolge des Dauerschlafs gestört wird. In Verbindung mit diesen Möglichkeiten sei auf die recht zahlreichen Literaturangaben verwiesen, sowie auf die eigenen früheren Erfahrungen, nach denen die Narkose — im Gegensatz zu den Feststellungen von BESREDKA [3] — bei bereits vorhandener Sensibilisierung die Symptome der lokalen und generalisierten anaphylaktischen Manifestationen nicht beeinflusst. Jedenfalls wurden von uns zur endgültigen Klarstellung dieser Frage Versuche angesetzt, in denen untersucht wird, wie der Dauerschlaf das durch bekannte Immunkörpermengen hervorgerufene passive ARTHUS—SACHAROW-Phänomen beeinflusst.

Auf Grund der bisherigen Ereignisse ist es aber als sehr wahrscheinlich anzusprechen, dass die Narkose die Immunkörperbildung bis zu einem gewissen Grade hemmt. Diese Annahme wird auch durch den Umstand unterstützt, dass sich die Immunglobuline während Phenyläthylbarbitursäure-Narkose erst nach mehrmals wiederholter Antigeninjektion im Blute so anreichern, dass sie die in den Kontrolltieren anwesende Menge erreichen, und dass bei der Hälfte der narkotisierten Tiere die Injektion von Pferde- bzw. Rinderserum mehrmals wiederholt werden muss, bis das ARTHUS—SACHAROW-Phänomen auftritt. All dies weist mit grosser Wahrscheinlichkeit darauf hin, dass während Barbitursäurenarkose der Eiweissstoffwechsel nur vermindert imstande ist, sich an die Umweltreize anzupassen und auf die makromolekularen Reizstoffe mit physiologisch adäquaten Schutzreaktionen zu antworten.

Zusammenfassung

1. Laut Versuche an 38 Kaninchen beeinflusste der Dauerschlaf in 17 Fällen die zur Ausbildung des ARTHUS-SACHAROW-Phänomens notwendige Zeit und Antigenmenge nicht in einer wertmässig erfassbaren Weise, während die Narkose an 16 Tieren das Auftreten dieser Erscheinung ausgesprochen verzögerte und an 5 Tieren einen Monat hindurch verhinderte.

2. Auf Grund der bisherigen Versuche ist es wahrscheinlich, dass die Immunkörperproduktion infolge des Dauerschlafes abnimmt und dass diese Abnahme den relativ niedrigen Immunkörperspiegel im Blut und bei der Hälfte der Tiere die Verzögerung des auf den an die Gewebe gebundenen Immunkörpern beruhenden ARTHUS—SACHAROW-Phänomens verursacht.

3. Da die gleiche Narkosebehandlung die Menge der im Blut zirkulierenden Antikörper ausnahmslos in jedem Falle verminderte, das auf den an die Gewebe adsorbierten Immunkörpern beruhende ARTHUS—SACHAROW-Phänomen dagegen nur in der Hälfte der Fälle hemmte, so ist es möglich, dass die Narkose neben der Hemmung der Bildung von Immunglobulinen auch auf die Verteilung dieser Immunkörper zwischen Blut und Geweben eine Wirkung ausübt. Die Bestimmung der an die Gewebe adsorbierten Immunkörpermenge ist also unumgänglich notwendig, wenn das Gebiet der Immunkörperbildung klargestellt werden soll.

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НЕРВНАЯ СИСТЕМА И ИММУНИТЕТ, VI.
ВЛИЯНИЕ ДЛИТЕЛЬНОГО СНА НА ФЕНОМЕН АРТЮСА—САХАРОВА

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Резюме

Согласно экспериментам, проведенным на 38 кроликах, длительный сон в 17 случаях не оказывал оценимого влияния на время, необходимое для развития реакции АРТЮСА—САХАРОВА, и на количество антигена, однако у 16 животных выраженно замедлял реакцию, а у 5 кроликов в течение одного месяца препятствовал появлению феномена.

На основании проведенных до сих пор опытов оказывается вероятным, что под влиянием длительного сна понижается образование антител и понижение это является причиной относительно низкого уровня циркулирующих в крови антител, а у половины животных запаздывания феномена Артюса—Сахарова, базирующегося на адсорбированных к тканям антителах. Ввиду того, что при одинаковом способе усыпления, титр циркулирующих в крови антител понижался в каждом случае без исключения, а появление феномена Артюса—Сахарова, базирующегося на адсорбированных к тканям антителах, понижалось только в половине случаев, возможно, что искусственный сон — наряду с торможением образования иммунных глобулинов — влияет также и на их распределение между кровью и тканями. Следовательно определение количеств адсорбированных к тканям антител является неизбежно важным для выяснения места возникновения антител.

PHAGE-TYPES OF S. TYPHI STRAINS ISOLATED IN HUNGARY AND RELEVANT INVESTIGATIONS MADE FROM 1950 TO 1954

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The discovery of the Vi-antigen contained in *S. typhi* [1] and the speedy recognition of its importance have induced numerous investigators to study the existence of bacteriophages that are specific to the Vi-form of the typhoid bacillus [2, 3, 4].

Outstanding results were achieved by CRAIGIE and YEN [5], who produced a preparation of the so-called Vi-phage II. This phage when propagated on typhoid strains of various origin but whose antigenic structure cannot be differentiated serologically, develops a specificity for the particular strain on which it has been grown. From this phage have been derived the other phages adapted for phage-typing, at present 33 in number, which make it possible to identify strains containing Vi-antigens that cannot be differentiated by the use of serological methods [6].

According to the data in literature, the Vi-phage-type of the *S. typhi* strains is practically stable and remains unaltered, even on human passage, or reveals differences of no appreciable consequence in practice. In other words, all strains isolated from outbreaks due to a common source of infection are of the identical phage-type. Therefore a carrier cannot be regarded as responsible for a given outbreak unless he is excreting the same phage-type strain. The practical significance of phage-typing has been substantiated by a great number of observations based on epidemiological evidence [7, 8, 9, 10].

The method of standard phage-typing as suggested at and adopted by the International Microbiological Congress held in Copenhagen in 1947 [11] is being introduced and used in epidemiological research in an increasing number of countries.

In Hungary, typing of the isolated strains was begun in December, 1948. As reported in 1950, 94 per cent of the strains examined up to that time corresponded to the international phage-types, while 6 per cent proved to be untypable; a percentage more or less in harmony with the pertaining data in the literature. In the same report an account was given of some investigations into sources of infection [12, 13].

In the present communication we wish to report our latest results and on the basis of our own findings, to deal with the problem of whether or not a strain excreted by the same carrier undergoes a change in phage-type as the years pass by.

Materials and methods

The Vi II type phages for phage-typing and the corresponding strains were obtained in 1948 from the International Enteric Reference Laboratory for Enteric Phage-typing (London). Since then the phages and the strains were stored at the prescribed temperature of + 4 °C. The strains were subcultured semiannually on DORSET egg medium. Both phages and strains are being tested at regular intervals. Phage-typing is carried out in conformity with the original description [11], the only difference being that horse flesh digest medium instead of the prescribed Hartley's beef-digest agar is used; a great number of comparative experiments having convinced us that horse flesh is equivalent to beef for this medium.

Since 1950, all laboratories of the country engaged in diagnostic work send their isolated *S. typhi* strains to our laboratory for phage-typing. In these laboratories for the isolation of *S. typhi* cultures LOVREKOVICH's bismuth sulphite agar is used. The blood cultures are plated on brilliant-green agar medium, and are identified on RUSSEL's three-sugar-semislant or on brilliant-green agar. The strains to be typed are cultured from one colony. (This diminishes the value of the findings, but the phage-typing of one colony indicates whether it will be necessary to examine more colonies from the original selective medium.) Strains derived from one colony are always plated on agar from which 10 to 20 «S»-colonies are then transferred to 2 ml of bouillon. If a strain is found to be degraded, or if the result of the test proves to be different from that of a preceding analysis, then 5 to 10 colonies are separately inoculated and adjusted for phage-typing, and at the same time a freshly isolated or the same stored strain is being asked for. This method repeatedly helped us to determine the phage-type of strains that in the first analysis had been found to be degraded. In some cases several colonies were isolated from the original elective media in our own laboratory. In the following, analyses of variations based on examination of several colonies will be indicated separately.

Results

Distribution of S. typhi strains in Hungary by phage-types

Table I shows the results of phage-typing during the period from 1950 to 1954. The figures indicate the annual percentage distribution according to foci of infection. We agree with FELIX [14] in that this picture gives a more accurate view of the distribution than that based on individual cases.

It can be seen from Table I that in the years between 1950 and 1954 the most abundant sources of infection were the foci with phage-types «El» (19,7%), «A» (17%), «Dl» (13,6%), and «Fl» (11,7%). Phage-types «G», «H», «K», «L1», «L2», «M», and «O» were not encountered, while the adapted standard phages «26», «27», «28», «29», «30», «31», «32», and «33» were not yet used for phage-typing in 1954.

There were no significant differences between foci of identical phage-types in the annual distribution, save in 1953 when the number of foci of the «Fl» phage-type amounted to 20 per cent as against 9,1 per cent in 1952 and 12,6 per cent in 1954. The reason of this fluctuation still awaits clarification.

Table I

Percentage distribution of phage-types according to foci in Hungary between 1950 *and* 1954

Vi-phage type	1950	1951	1952	1953	1954	1950—54
A	21,6	14,9	14,4	17,9	15,1	17,0
B 1	0,6	0,5	0,4	0,2	0,3	0,4
B 2	4,1	4,3	2,5	4,1	3,5	3,8
B 3	0,8	3,5	0,4	0	1,1	1,3
C	6,6	9,3	8,3	6,1	5,4	7,3
D 1	12,8	13,6	15,5	13,6	12,5	13,6
D 2	1,3	0,6	1,5	1,0	1,1	1,1
D 4	1,1	0,9	0,2	1,2	0,3	0,8
D 5	0	0,3	0	0	0	0
D 6	0,2	0,2	0,2	0	0	0,1
E 1	19,7	18,7	20,1	16,2	25,9	19,7
E 2	0	0	0,2	0	0	0
F 1	8,3	10,0	9,1	20,3	12,6	11,7
F 2	0	0,2	0,4	0	0,3	0,1
J	0,2	0,2	0	0,4	0	0,1
N	0,8	2,5	1,7	1,8	0,3	1,5
T	1,3	0,5	1,1	0	0,5	0,7
X*	0,3	0,2	1,1	0,6	0	0,4
Degraded Vi strains	7,4	7,4	9,8	6,9	10,5	8,2
Untypable Vi strains	8,8	9,8	8,6	8,5	7,0	9,1
Vi-negative cultures	4,1	2,4	4,5	1,2	3,5	3,1
Number of cultures analyzed	1287	1256	1178	1208	1475	6404
Number of persons examined	970	1005	837	925	653	4390
Number of foci	634	631	472	493	371	2601

* Phage «X» was adapted in Hungary in 1949; strain «X» is probably a variant of strain «E1». Final identification of phage «X» in progress.

The number of foci is not quite reliable, for while one focus may have given rise to outbreaks in several places, each of them is accounted for as a separate focus because the source of infection has not been detected.

The predominance of foci with any particular phage-type in a country is always determined by the phage-type of the carriers left over from previous epidemics. This gives at the same time a clue to the phage-types and the proportions of earlier epidemics.

Table II shows the percentage distribution of the registered carriers in Hungary, according to phage-types, and, for the sake of comparison, once

more indicates the percentage distribution in the years from 1950 to 1954 of the foci of infection, according to phage-types.

Table II

Percentage distribution according to phage-types of carriers registered in Hungary, as compared with the percentage distribution according to phage-types of foci of infection between 1950 *and* 1954

Vi-phage-type	Carriers	Foci
A	20,1	17,0
B 1	0,2	0,4
B 2	4,0	3,8
B 3	0,6	1,3
C	7,1	7,3
D 1	15,7	13,6
D 2	1,0	1,1
D 4	0,7	0,8
D 6	0,6	0,1
E 1	22,4	19,7
F 1	12,8	11,7
F 2	0,6	0,1
J	0,2	0,1
N	1,2	1,5
T	1,0	0,7
X	0,4	0,4
Degraded Vi strains	4,2	8,2
Untypable Vi strains	5,7	9,1
Vi-negative cultures	1,5	3,1
Number of carriers examined	1201	—
Number of foci	—	2601

It is only natural that the figures in the two columns are in agreement. Infections departing from carriers, it was to be expected that in a country like Hungary, where the carriers detection has been carried out over a considerable number of years, the phage-type distribution of undetected hosts should not significantly deviate from that of recorded carriers. This is further supported by the history of the newly discovered carriers whose phage-type distribution was essentially conforming to that of the earlier discovered cases.

Table III shows the predominant and rarely occurring phage-types in some European countries and in Indonesia (Djakarta). (Excepting those for Hungary,

the data were taken from the reports of the 5th meeting of the International Committee for Enteric Phage-typing held in 1950, and the 6th International Microbiological Congress held in 1953.)

Table III

Dissemination of phage-types encountered in Europe and Indonesia (Djakarta)

	Most frequent phage-types in the order of their frequency	Rare phage-types
Austria	El, Dl, A, C	—
Czechoslovakia**	El, Dl, Fl, A	—
Denmark*	A, Fl, El, C	—
France	C, E1, A, Dl	M
Holland*	El, A, Dl, C, Fl	D6, G
Ireland*	El, A, Dl, C	—
Yugoslavia*	A, El, Fl, Dl, C	D6
Poland**	El, Fl, A	—
Hungary*	El, A, Dl, Fl	D6, J
Great Britain*	El, A, C, Dl	D6, E2, G, J
Norway*	El, C, Fl	J
West Germany*	El, Fl, A, Dl, C	J
Italy		
North*	A, Dl, C, El, N	D5, D6, E2
Middle*	C, El, Dl, A, Fl	D6, H, J
South*	Dl, A, C, El, N	D6
Portugal*	B3, A, El, T, Dl	—
Indonesia (Djakarta)	D6, A, D2, El	E2, J, M

* Phage-types in focal distribution.

Unmarked countries, further Czechoslovakia and Poland, based the figures for the distribution of phage-types on individual examinations of patients.

** Data taken from the report of the 5th International Microbiological Congress of 1950.

Data regarding other countries — except Hungary — adopted from the report of the 6th International Microbiological Congress of 1953.

Table III shows that in 9 out of 14 European countries, including Hungary, it is type «El» that occupies the first place among the predominant ones. It also shows that the distribution of the other dominant phage-types is fairly the same over all the European countries. The rare occurrence of «D6» merits special attention. In Europe it has been isolated in Hungary, England, Italy, Holland and Yugoslavia, while Indonesia stands alone, even among the non-

European countries, for having it as the dominant type. None of the chronic carriers who had had typhoid fever before 1944 has been found to be of the «D6» type. This appears to permit the conclusion that the type had been imported to Hungary by the military that marched through the country that year. Among the subjects who had developed the disease in 1944, it has actually been possible to find a carrier responsible for the grave epidemic in 1947 [12]. It is safe to assume that in the other European countries too, it is a recent acquisition and not a type left over from earlier epidemics. Another phage-type imported to Hungary is the «J» type. Its carrier had had the disease in 1944, and has proved to be the source of a few infections in the course of the last years.

A special interest attaches to these recently imported phage-types for the reason that their sources can be more readily determined and their dissemination more easily followed, than those of infections originating from earlier epidemics.

Variations in the specificity of strains

It has already been mentioned that a great number of investigations based on ample evidence substantiated the stability of the phage-types and, in accordance therewith, the epidemiological significance of typing. Several predominantly theoretical problems have nevertheless remained unsolved [11]. Naturally, theoretical questions not infrequently have bearings on practical difficulties. It was recognized already in the initial stages of phage-typing that, on the one hand, there exist degraded strains whose occurrence introduces an element of uncertainty into the tracing of the source of infection [11, 15]; on the other hand, that sometimes a strain of two distinct phage-types can be isolated from cultures, especially if the latter had been maintained in the laboratory for a considerable period of time [11, 16].

Degraded strains exhibit anomalous sensitivity in various degrees to several type phages, obscuring these by their original phage-type and rendering source detection futile. Degradation accordingly means the possibility of variation in the phage sensitivity of the strains; on the other hand, the fact that a culture may yield strains of two different phage-types, is not conductive to a reassuring solution of the problem of type variations [15, 17].

The question under review has been investigated by many workers. FELIX and ANDERSON suggested the existence of a close correlation between the development of degraded strains and the latent phages [18, 19], and claim the possibility of a degradation of *S. typhi* in the organism of chronic carriers of long standing [20]. They described a hospital outbreak caused by a degraded strain, and emphasized the difficulties in detecting the source of infection [21]. They have demonstrated that a considerable part of the *S. typhi* strains are

lysogenic, and that their phage-type depends on the phages that have corresponding prophages in the strain (Gamma phage, latent phage) [22, 23, 24]. In the course of their experiments they reproduced changes *in vitro* which, though infrequently, occur under natural conditions; in addition, they produced variations which, though not yet actually encountered, may assumedly occur in nature [18, 19, 20, 22, 23]. JUDE, NICOLE and DUCREST likewise reported similar experiments [25].

Being aware of the possibility of these variations, it was from the outset part of our working programme to test, as far as possible annually, the phage-types of our recorded carriers with a view to following up the changes in these types occurring within the organism, and to observe their frequency. To our knowledge, methodical observations of this kind extending over a longer period have not been reported in the literature.

Strains obtained from 850 carriers have been subjected to examination on 2200 occasions since 1950. There are 347 among these carriers whose strains had been analyzed as early as 1948 or 1949.

The figures in Table IV show for how many years since 1950 each individual carrier had been typed, as also the total number of typings.

Table IV

Serial examination of carriers

Examined in	1	2	3	4	5	Total of 5 years
	years					
Number of persons	147	437	195	36	10	825
Number of examinations	219	985	695	171	91	2159

The carriers in the first column have also passed through previous examinations, as their phage-type had already been determined prior to 1950.

The results of the serial investigations extending over 5 years are summed up in the following.

There were 99 carriers out of a total of 825 from whose secretions we isolated strains differing from those obtained on a previous occasion.

1. Cultures of degraded strains were obtained from 75 carriers.

a) Of these, 30 carriers were found to belong to phage-type «A» at the first analysis, while the strains isolated on subsequent occasions revealed some sensitivity to an increasing number of various phage-types, the cultures from 3 of these carriers having become at last completely refractory to Vi-phage II.

b) While found to be degraded when first analyzed, the strains obtained from 15 carriers proved to belong to the «A» group in later isolations.

c) There were 4 carriers whose culture was now of phage-type «A», now degraded.

d) There were 26 carriers whose strains had been first sensitive to one of the phages other than the «A»-type and become degraded in subsequent isolations or, reversedly, had been degraded at the first test and were found to be sensitive to one of the phage-types, except «A», on later occasions.

2. We found 24 carriers who belonged to a definite phage-type on one occasion, and to a definitely different type on another occasion.

a) In 8 cases investigations and control tests supported the conclusion that the serial appearance of two different phage-types was to be ascribed to processes in the organism of the carrier.

b) The possibility of the strains having been confounded could not be excluded in 16 of the cases. (Phage-typing was repeated with several colonies of the subculture, and fresh material for analysis was asked for. It has not yet been cleared whether a mistake occurred or an actual change had taken place in the sensitivity of the strains.)

The following is a description of the confirmed changes in phage-type variations.

From the epidemic of the «D6» phage-type in 1947, five carriers are kept in evidence. Beginning with 1949 and 1950, their repeatedly isolated strains were subjected to several examinations. Until 1951, no change was found in the specificity of the strains ; they belonged invariably to the «D6» phage-type and were negative to xylose. Then, in 1951, a strain isolated from the faeces of one of the carriers (No. I) proved to be of the phage «A» type. Suspecting a confusion of the strains, the analysis was repeated ; having obtained the same result from the second test, fresh faeces were asked for, but at the same time the above mentioned strain was plated. Ten of the colonies from the plating were each separately adjusted for phage-typing. Eight of them were found to belong to the «A», and two to the «D6» type. The faeces submitted was so worked up that the colonies isolated from the selective medium were separately adjusted for phage-typing. Four of the isolated 5 colonies were found to belong to the «D6», and 1 to the «A» type. Ten colonies each were isolated and examined from the third and fourth consignment of material. All of them proved to be of the «D6» phage-type. It should be noted at this point, that the tests were repeated several times with the cultures obtained on the first three occasions. The majority of the colonies in the culture derived from the first material was of the «A» phage-type, that in the culture from the second material of the «D6» phage-type, while nothing but «D6» colonies were found in the culture from the third material. The cultures originating from this carrier have been found to be of the «D6» phage-type ever since, but the phage-type underwent some change in character inasmuch as it now reveals sensitivity to the «C» phage as well [26].

Similar changes were observed in the case of another carrier (No. II). He had had the disease in 1931. Strains isolated from his faeces were found to be of the «A» phage-type both in 1948 and 1951, while a culture in 1953 contained a «D1» type strain. On repeating the tests in the same year by analyzing several colonies taken from the selective culture medium, we obtained strains of the «A» and «D1» type, together with degraded strains. Strains were isolated on three more occasions in the course of 1953 ; they were of the «D1» type on the first, of the «A» type on the second, and degraded on the third occasion.

The third carrier (No. III), from whose faeces two different types of strains were isolated, had had typhoid fever in 1938. His strains were first typed in 1950 and found to be «D1». Three further isolations were made between 1950 and 1954, and there appeared no change in the phage-type on either occasion. When testing the faeces of this carrier in 1954, that is for the fifth time, the strains proved to be of the «A» phage-type. Since then, the faeces have been analyzed six times including two occasions on which isolated colonies taken from the selective culture medium were, each, separately examined. The strain obtained at each of these tests, whether based on one or five colonies, always belonged to only one type at a time ; in the last tests only «D1» types were obtained in September, and only «A» types in December, 1954.

Efforts have been made to establish on an epidemiological basis whether any disease had been caused by the three carriers during the five years. The data obtained showed no infections attributable to them.

Two carriers of the «F1» and «F2» phage-type were detected in the course of the serial analyses. One of them (No. IV) had had typhoid fever in 1914, and the strain excreted by him was first analyzed in 1949 and then in 1950 and 1952. On all occasions it was found to be of the «F1» phage-type. Subsequent isolations made in 1953, 1954 and 1955 yielded strains of the «F2» type. Our epidemiological records show this carrier to have been responsible for one infection in 1953. Strains isolated from the excreta of the infected patient proved to be of the «F2» phage-type.

Carrier No. V, whose history contains no mention of typhoid fever, figures in our records since 1949. He excreted a strain of the «F2» phage-type in 1949. There occurred two outbreaks in 1949 and 1950 which may have been caused by this carrier. Both infected patients excreted a strain of the «F2» phage-type. The carrier himself had not been tested again until 1954, when the strain isolated proved to be of the «F1» type. According to our epidemiological records he was responsible for one more infection ; the patient in that case excreted a strain of the «F1» type.

Variation occurring within the «D» type requires close attention [26]. Our serial tests revealed one carrier (No. VI) who, in addition to the «D1» type, had first excreted a strain of the «D4» phage-type and later one sensitive to both «D1» and «D4» type-phages. HENDERSON and FERGUSON [27]

were the first to report the joint appearance of the «D1» + «D4» phage-types. Some time ago there was an academic discussion in progress about whether it is justified to regard «D1», «D4» as a separate type.

Two more carriers (Nos. VII and VIII) had proved first of the «D1» type, while later the isolated *S. typhi* strain proved sensitive to the «D1» + «D4» phages. The existence of an epidemiological correlation between strains of the «D1» type and those sensitive to «D1» + «D4» type-phages seems to be corroborated by the established facts that (i) two carriers who excreted a «D1» type strain, infected each a patient from whose material a strain sensitive to «D1» + «D4» was isolated, and that (ii) of two patients infected from the same source one excreted a strain of the «D1» phage-type, and the other a strain that was sensitive to the «D1» + «D4» type-phages.

Practical examples demonstrating the epidemiological significance of phage-typing

1. In 1950, there had occurred two cases of typhoid fever in a village. The patients had belonged to the «E1» phage-type. No search for the source of infection had been made at the time. Three more cases were reported from the village in 1955, the patients belonged likewise to the «E1» phage-type. A carrier from the same village had been kept in evidence since 1935 ; no S. typhi had been identified from his excreta since a considerable length of time ; his strain had remained untyped. Though unable to trace the said infections to this carrier, the physician responsible for the field work ordered serial analyses to be made of the faeces of the carrier, with a view to determining the phage type of his strain. At the same time, an independent search was instituted, taking the «E1» type patients of 1950 as points of departure. The results were as expected. The strain of the recorded carrier was not of the «E1» phage-type, while the search for the source of the 1950 infections revealed a carrier of the «E1» type to have been undoubtedly responsible for the outbreaks both in 1950 and 1955.*

2. Three carriers are on our records from another village. All of them belong to the «D1» phage-type. No infections were reported from the village for a number of years, while a mild epidemic broke out in the neighbourhood. The strains isolated from the patients were of the «D1» phage-type. One of the patients had the same surname as one of the carriers in the adjacent village, who turned out to be his sister. Inquiries revealed that some weeks before he had been present at an entertainment given by his sister at her farmstead.

3. One of our recorded carriers, kept in evidence since 1935, belongs to the «F1» phage type. Strains were isolated from his secreta three times since 1950 and their phage-type was always found to be the same. Infections were reported in 1952 from the village where the carrier lived. The cultures obtained from the patients proved to be of the «A» phage-type, so that the responsibility for the disease was averted from the recorded carrier. A research after the source of infection revealed the existence of an unregistered carrier who had come a short while before to live in that part of the village where the outbreak occurred.**

4. Strains of the «D2» phage type were isolated from the faeces of an infant in Budapest who suffered from typhoid fever. This type is comparatively rare in Hungary so that our records of the occurrence of strains of this phage-type were easy to survey. Enumerating the names of villages from which carriers of the «D2» phage-type figured on our lists, we asked the family if the baby had been taken to one of these places prior to the disease. Surprised to hear the second of the enumerated village names they told us that the baby had been brought home from that village in an apparently healthy condition a few days before becoming ill. They were still more astonished on hearing the names of persons who, — according to our records — had

** We are indebted to *Dr. P. Kneffel, Chief Medical Officer of the Station of Hygiene and Epidemiology in Szombathely, as also to **Dr. J. Szita, of the Department of Bacteriology of the State Institute of Hygiene in Budapest, for the data concerning source detection.

had typhoid fever some years ago and whose strains belonged to the «D2» phage type. It was revealed that two of these persons lived in the house in which the baby stayed as a guest of his grandmother, while one of the recorded «D2» carriers, who lived on the opposite side of the street, paid repeated visits to the grandmother and repeatedly attended to the baby.

Discussion

Phage-typing has often been found to be of great assistance in tracing sources of infection.

Our work in this respect began under favourable conditions. Phage-typing is especially effective as an aid in source detection when the infections occur sporadically : and typhoid fever had become mostly sporadic in Hungary by the time our investigations were started. The success of our work was due partly to the fact that carriers have been registered in Hungary ever since 1930, so that the records kept at the State Institute of Hygiene were of a great value in epidemiological research even before the introduction of phage-typing.

Within certain limits, phage-typing helps to identify the strains coming from a common source. Work of this kind is greatly facilitated by epidemiological records and the registers containing the phage-types of carriers and persons who had typhoid fever since 1949.

Our epidemiological records make it also possible to follow up the development of degraded strains. According to the records, degraded strains were obtained from the faeces of 75 carriers. It would seem obvious to infer that should these carriers become the causes of outbreaks, phage-typing would be of no avail in the detection of the source of infection. Actually, the situation is a different one ; for, comparisons made of the phage sensitivity of the degraded strains, duly considering its degree as well as the sensitivity to the individual type phages, will in quite a number of cases permit of concluding to the common source of infection.

It has turned out to be a further facility that ever since our work was started careful records have been made of the variants we were able to detect within the international phage-types. To this category belong the strains which, besides the type phages in the «D» group, are also lysed by the «N» or «N» + + «L2» types [26]. NICOLLE and HAMMON [28] regard the «N + D1» type as a separate one. While studying infections originating from a common source we too encountered variants of this kind. It was found that the identical variant may occur repeatedly within one particular focus. This finding is duly taken into account in our search after sources of infection. A study of the relative epidemiological bearings is in progress. We have, for the time being, allocated such variants to the «D1» group.

Although variations in the phage-type of strains have been found to cause temporary difficulties, they by no means diminish the value of phage-

typing in practical epidemiology. Besides, many of contradiction that arose in practice has been cleared since we began our routine analyses in 1948.

It has been mentioned that our carrier No. I who belongs to the «D6» phage-type excreted on two occasions strains which proved to be of the «A» phage-type. A similar phenomenon observed *in vitro* has been reported in the literature. In a communication published in 1953, FELIX described that a strain of the «D6» phage-type had been isolated from the faeces of a patient in 1951. After storage for one year in a dark place at room temperature, there appeared colonies of the «A» type in the subculture of the strain. This «A» variant proved to be easily transformable into a phage of the «D6» type by means of the latent phage of «D6» [20]. Taking this into account, there is no need to suppose that our carrier No. I acquired a fresh infection ; the less so as there is no epidemiological evidence for such a supposition. It is more likely that the variation in type due to the loss of the latent phage took place within the organism of the carrier during the investigations, and that it was a process similar to the one observed by FELIX *in vitro*.

It is not known to what phage-type the strains belonged which had originally been excreted by carriers II and III. Carrier No. III may be assumed to have been infected by a strain of the «D1» phage-type, for two other carriers in the village, one of whom had had the disease in 1930 and the other in 1948, likewise excrete a strain of the «D1» type. No carrier of the «A» type has been recorded in the village.

Our observations concerning carriers II and III appear to be analogous to those made by CRAIGIE in the course of his experiments in which he succeeded, as long ago as 1946, in transforming a strain of the «A» phage-type into one of the «D1» type.

HELMER was the first to isolate strains of the «F1» and «F2» phage-types from the faeces of the same patient [29]. The epidemiological identity of these two types was obscure until 1951. Careful experiments made by ANDERSON [23] proved the existence of an epidemiological relationship between the types in question, and he also succeeded in transforming these types into each other *in vitro*. Our serial investigations have confirmed (v. carriers Nos. IV and V) on an epidemiological basis the capability of the «F1» and «F2» types to transform from the one into the other; a moment to be given consideration in carrier detection.

We suggest that variations in type similar to those observed *in vitro* [20, 22, 23, 25], are not uncommon in nature. There may be differences dependent on whether typhoid fever is endemic or sporadic in the area where tests are condocted.

In our view, the major problem in connection with the epidemiological significance of variations in type is to ascertain the conditions in the organism conducive to the loss of, and infection by, the latent phage or phages, and to

elucidate the factors which, after the loss of the infection, promote the predominance of the new phage type or the disappearance of the original one.

Summary

1. The S. typhi strains isolated in Hungary are such as can be ranged with the international phage types. The following are the types occurring in the country: «A», «B1», «B2», «B3», «C», «D1», «D2», «D4», «D5», «D6», «E1», «E2», «F1», «F2», «J», «N», «T», and «X». In addition, strains also occur which represent certain variations and display sensitivity to «D1» + «D4» «D1» + «D2» + «D4» + «D5» + «D6» + «N», as also to «D1» + «D2» + + «D4» + «D5» + «D6» + «N» + «L2» phages. Of the isolated strains, 9,1 per cent are untypable, and 8,2 per cent degraded. The distribution of the phage-types is similar to that in the other European countries.

2. The results obtained have tended to show that, although it is a rare occurrence, the phage type of a strain isolated from the same carrier may vary within the organism with the passing years.

3. Under Hungarian conditions, too, phage-typing is a valuable aid in epidemiological investigations.

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ТИП ФАГА ВЫДЕЛЕННЫХ В ВЕНГРИИ ШТАММОВ S. TYPHI И СВЯЗАННЫЕ С ЭТИМ ИССЛЕДОВАНИЯ В 1950—1954 гг.

М. ЁРШИ

Р е з ю м е

1. Изолированные в Венгрии штаммы S. typhi могут быть отнесены к международным типам фага. В Венгрии встречаются следующие типы фага: «A», «B1», «B2», «B3», «C», «D1», «D2», «D4», «D5», «D6», «E1», «E2», «F1», «F2», «J», «N», «T», «X».

Встречаются кроме того типы представляющие некоторые вариации, чувствительные к фагам «D1», + «D4», «D1» + «D2» + «D4» + «D5» + «D6 + N», а также к фагам «D1» + «D2» + «D4» + «D5» «D6» + «N» + «L2», 1% выделенных штаммов не могли быть типизированы, деградировалось 8,2%. Распределение типов фага подобно таковому в других европейских странах.

2. Согласно нашим исследованиям нужно считаться с тем, что тип фага штамма, происходящего от одного и того же бациллоносителя, в течение лет может измениться внутри организма, хотя это бывает очень редко.

3. Определение типа фага оказывает ценную помощь для эпидемилогических исследований также и при наших условиях.

THE ANTIBODY CONTENT OF IMMUNE SERA ABSORBED WITH INFLUENZA VIRUS, AS DETERMINED BY DIFFERENT SEROLOGICAL TESTS

By

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The antibody produced in the living organism against influenza virus as an antigen is usually demonstrated by the complement fixation (CF) haemagglutination inhibition (HI) and neutralization tests. Under certain experimental conditions the following techniques may also be used: the precipitation test (KNIGHT [1], JENSEN and FRANCIS [2]) the agglutination test (TAKÁTSY [3]), the conglutination test (STOKER and MARMION [4]), as well as the indirect complement fixation, or complement fixation inhibition (CFI) test.

If the antibody content of an immune serum is determined on the basis of various tests, the results are often not unequivocal, *i. e.* there will be more or less pronounced discrepancies between the results yielded by the different tests. For example, when the *in vitro* HI test was compared with the *in vivo* neutralization test, in the mouse by STUART-HARRIS and MILLER [5], and in eggs by HILLEMAN and HORSFALL [6], marked discrepancies were found between the antibody titres *in vitro* and *in vivo*. Similar differences between the above and the CF tests have been observed by WIENER and HENLE [7].

In our earlier studies [8, 9, 10] the conclusions regarding the antigenic structure of the influenza virus had been drawn from HI tests made in absorbed sera. Although the HI test, by virtue of its specificity is well suited for use in such studies, it seemed still desirable to carry out parallel with the HI tests also neutralization tests, considering that neutralization seems to be a more intrinsic property of immunity than is haemagglutination inhibition. The experiments were supplemented also by parallel CF tests or, in chicken sera, by CFI tests.

Materials and methods

Virus strains: Influenza A PR8, Melbourne (Mel); influenza A-prime Paris P. L. 1/49, England 1/51 (Lp), and Budapest 4/49 (4/49).

Immune sera. Immune sera produced in the hamster and in the chicken were used. The hamsters were given two intramuscular injections, separated by an interval of two weeks, of an emulsion of purified virus in oil (Bayol F and Arlacel A). FŰRÉSZ [12] has namely successfully immunized hamsters by SALK and LAURENT method [11]. Three weeks after the second inoculation the hamsters were killed by bleeding. The chickens were also inoculated with a

purified virus suspension, 2 to 3 times at 2-day intervals, and then again 6 to 8 weeks later. After two further weeks the chicken were killed by bleeding.

The *haemagglutination*, the *haemagglutination inhibition* (HI) and the *complement fixation* (CF) tests were performed by the spiral loop micro technique [13]. In the CF tests purified virus suspension, *i. e.* somatic antigen was used.

Complement fixation inhibition test (CFI test). To 0,025 ml of serum diluted by means of the loop in the grooves of a plexiglass plate, was added one drop (0,025 ml) of antigen, and, after 30 minutes of incubation at 37 °C, one drop of complement and one drop of positive hamster serum were added. After another 20 minutes of incubation one drop of the haemolytic system was added to the mixture. In practice, there were 3 rows of dilutions for each test serum. A stock dilution of antigen, prepared according to the result of a preliminary titration, was added to the first row, while to the second twofold, and to the third row fourfold dilutions of the stock antigen were added. Titration rows not giving complete fixation at their end were ignored, and of the others the titre of serum was accepted on the basis of that row in which the dilution of antigen was the highest. The positive hamster serum was used at a concentration four times higher than that giving a + + reaction in the simple CF test.

The neutralization test was carried out essentially by the roller tube tissue culture technique described by HORVÁTH [14]. Twofold dilutions were made of immune serum and 0,025 ml of each dilution were added to each of four tubes. An equal volume of virus diluted to contain 10, 100, and 1000 ID50, respectively, was added to the dilutions of serum. After 1 hour at 4 °C, minced chorio-allantoic membrane from eggs incubated for 15 days and 0,5 ml nutrient fluid were measured in by means of an automatic pipette, stirring the mixture continuously. After 72 hours at 35 °C the nutrient fluid was titrated for HA. Prior to testing them for neutralization, the absorbed sera were kept at 56 °C for 30 minutes to inactivate residual virus.

The technique of the *immune serum absorption test* will be described in the section «Experimental.»

In order to facilitate comparison between the different reactions, the antibody titre of sera was expressed by the formula

$$t = 4n_t + p,$$

where

n_t = number of serum dilutions giving complete reaction (++++) in a titration row

p = degree of reaction in samples showing partial reaction, expressed in crosses (+)

Since in the neutralization tests there were four parallels for each dilution of serum, the value of p is expressed as the number of tubes showing complete neutralization among the total of four.

Experimental

Hamster and chicken sera were used. Sera from both species have the advantage that they contain only negligible amounts of non-specific inhibitor. The hamster serum has the further advantage of giving the CF test too. The chicken serum was employed in order to facilitate comparison with our earlier results. Since, however, the latter is unsuitable for use in the CF test, it was tested for CFI.

In the first series of experiments, unabsorbed immune sera were examined for homologous and heterologous titre by the HI, neutralization, CF and CFI tests. The results expressed by «t» (*v.* in the section «Materials and Methods»), and »d_1« values (difference between homologous and heterologous t values) are shown in Table I.

The data in Table I reveal that immune sera by the CF test, the hamster and chicken immune sera by the CFI tests gave, in general, lower homologous titres than by the HI and neutralization tests. The difference between the

Table I

Homologous and heterologous titres of hamster and chicken immune sera, as determined by different techniques

Serum	Animal	Virus	HI		Neutralization		CF		CFI	
			t	d_1	t	d_1	t	d_1	t	d_1
PR 8	hamster	PR 8 Mel	37 30	7	39 23	**16**	**29** 24	**5**	—	—
Mel	hamster	Mel PR 8	40 40	0	34 34	0	32 32	0	—	—
4/49	hamster	4/49 Lp	40 36	4	41 34	7	38 37	1	—	—
Mel	chicken	Mel PR 8	43 40	3	38 37	1	—	—	34 26	8
Paris	chicken	Paris Lp	40 40	0	38 32	2	—	—	30 26	4
4/49	chicken	4/49 Lp	36 34	2	43 40	3	—	—	26 18	8

corresponding «t» values was as high as 8 with the hamster, and 16 with the chicken sera, corresponding to four respectively sixteen times lower titres. The differences in titre between the HI and the neutralization tests were usually lower, but a threefold difference in titre($t_{HI} - t_{neutr} = 6$) also occurred. The difference between homologous and heterologous titres (d_1) was the highest by the neutralization test with hamster sera and by the CFI test with chicken sera, suggesting that these tests possess a higher specificity.

In the second series of experiments, in which the antibody content of absorbed sera was examined simultaneously by the various tests, it had to be ascertained whether not even traces of the virus-antibody complex had remained in the absorbed sera, since being able to combine with complement they would have interfered with the CF reaction. Subsequent filtration or ultracentrifugation presented technical difficulties, so that the carbon adsorbed virus preparation used in the previous experiments [15] was employed. The purified and concentrated preparations of virus were adsorbed unto active natural gas soot. This kind of carbon adsorbs about four times more virus than common medical charcoal and the adsorption is so firm that the virus cannot be eluted, but still it loses practically none of its antibody-binding capacity. The virus-binding capacity of soot was determined and it was found that 1 mg of carbon could adsorb a quantity of virus equivalent with that contained in 1 ml of a virus suspension with a HA titre of 1 : 6000. The carbon suspension was rendered adequately homogeneous and completeness of adsorption was provided for by

exposure to ultrasound (600 megacycles, 2 W/square cm, for 3 minutes). The carbon-virus preparation was then washed two or three times and taken up in known volume of saline. A determined amount of the preparation wars centrifuged and the serum to be absorbed was added to the sediment. After 15 minutes of shaking and further centrifugation the supernatant was drawn off and tested for antibody content.

Table II shows the homologous and heterologous «t» and «d_1» the values yielded by the HI, neutralization and CF tests carried out with hamster antisera to two strains (4/49 and Paris), following absorption with 3 different quantities of Liverpool virus.

Table II

Homologous and heterologous titres of hamster sera absorbed with Lp virus, as determined by different techniques

Serum	Carbon adsorbed virus mg	Virus	HI		Neutralization		CF	
			t	d_1	t	d_1	t	d_1
4/49	20	4/49 Lp	32 0	32	35 0	35	35 31	4
	10	4/49 Lp	34 13	21	35 0	35	39 35	4
	5	4/49 Lp	38 24	14	35 25	10	39 35	4
Paris	12	Paris Lp	22 0	22	29 0	29	24 16	8
	6	Paris Lp	24 0	24	34 0	34	24 22	2
	3	Paris Lp	28 16	16	32 13	19	31 27	4

The data in Table II reveal that, in contrast with the data in Table I, here the CF tests did not yield values lower than those obtained in the other types of test. The «d_1» values for the CF test were, however, remarkably low. This results from the fact that the same absorbed sera showing no heterologous titres in HI and neutralization tests gave a significant «heterologous» titre in the CF test. Since, however, the antibody reacting with the heterologous virus in the HI and neutralization tests had been completely absorbed, it is thought that the heterologous titre revealed in the CF test originated from the antibody that in the other two tests had only reacted with homologous virus. According to this, homologous antibody and heterologous virus would

form a loose, readily dissociable combination capable of fixing complement, but not strong enough to bring about complete absorption of the antibody by the virus, or to enable the antibody to prevent the virus from exerting its haemagglutinating and infective action.

The tabulated data obtained with the 4/49 hamster serum absorbed alternatively with 3 different quantities of virus have been represented also graphically, indicating the values of «d_1» by arrows. Fig. 1 illustrates clearly the homologous and heterologous titres at different degrees of absorption,

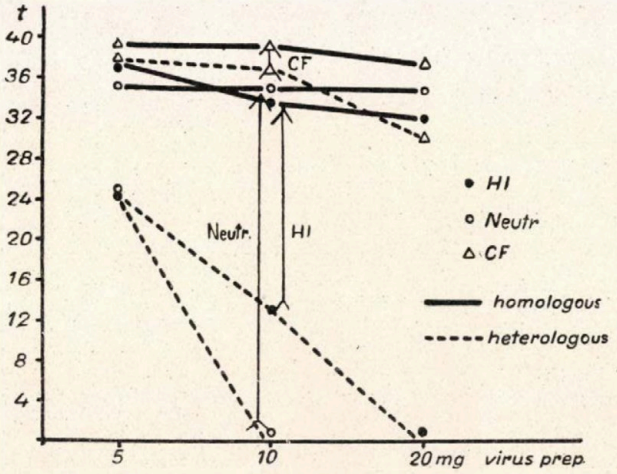

Fig. 1. Homologous and heterologous titres of hamster serum 4/49 after absorption with Lp virus as determined by different techniques

while the specificity of the simple tests is characterized by the length of the corresponding arrow.

In Table III are presented the data of a similar experiment, in which chicken immune serum was used and the CFI test was performed, instead of the CF test.

The data in Table III reveal that the CFI test is as specific as, or presumably in single cases even more specific than, the other two reactions. In some samples of serum namely the CFI test gave no heterologous reaction, although the other two types of test did indicate the presence of heterologous antibody. The difference in specificity between the three reactions, however, is not significant.

Discussion

On the basis of the experimental findings and in agreement with data in the literature, we, too, could demonstrate that the correlation between antibody titres differs with different influenza immune sera depending on the type of test used.

Table III

Homologous and heterologous titres of chicken sera absorbed with Lp virus, as determined by different tests

Serum	Carbon adsorbed virus mg	Virus	HI		Neutralization		CFI	
			t	d_1	t	d_1	t	d_1
4/49	18	4/49	20	20	25	25	15	15
		Lp	0		0		0	
	9	4/49	24	16	24	12	16	16
		Lp	8		12		0	
	4,5	4/49	32	4	31	4	24	8
		Lp	28		27		16	
Paris	20	Paris	16	16	20	20	12	12
		Lp	0		0		0	
	10	Paris	28	16	24	17	11	11
		Lp	12		7		0	
	5	Paris	36	12	30	7	16	10
		Lp	24		23		6	

As far as the specificity of the single tests is concerned, comparative examinations showed the neutralization and CFI tests to be the most specific although the HI test lags not too far behind them. Thus, these types of test are all suitable for differentiation of strains. The CF test, however, is less suitable for this purpose. It is easy to understand the divergence between the specificity of the CF test and that of the CFI test, if we realise that the latter does not indicate fixation of complement, but more of a reaction like serum absorption, except that the test measures not the residual antibody, but the excess of antigen.

In the immune serum absorption experiments the neutralization and CFI tests yielded results essentially identical with those obtained by the HI test, inasmuch as when absorption with the heterologous strain was continued until the residual antibody did not react any more with the absorbing virus in the HI test, the serum did not react with that virus in the neutralization and CFI tests, either. It can, therefore, be assumed that the neutralization and CFI tests, too, would have supplied the same results concerning the antigenic structure of influenza virus as had HI test in our previous studies.

The data in the literature have lent support to the view that a variety of antibody is produced against the influenza virus. In absorption experiments WIENER and HENLE [7] found that different antibodies were produced against the soluble and the particle antigens. On the basis of the discrepancy between the neutralization and HI titres of immune sera, BURNET and BEVE-

RIDGE [16], as well as STUART-HARRIS and MILLER [5] have arrived at the conclusion that the two kinds of antibody are not identical. The absorption experiments by WALKER and HORSFALL [17] support this latter view, since in the course of absorption the neutralizing titre was reduced to a significantly greater extent than the HI titre. We, too, made similar observations in some experiments (Fig. 1), but the occurrence of this phenomenon appears to be an exception rather than the rule. It must, however, be noted that in our experiments prolonged immunization was carried out, while WALKER and HORSFALL worked with sera of animals which had been given a single inoculation only.

To explain both our results and the data in the literature we must not even accept the conclusions drawn by SALK and LAURENT [11] from their experiments with vaccine in oil, according to which the different tests would indicate entirely different antibodies, to which there would be well-defined part-antigens in the influenza virus. The discrepancies between the different serological techniques may be explained also in the following way. As the virus is being broken down, a qualitatively very broad spectrum of antibody molecules develops. The different molecules will combine with varying intensity with the homologous and the heterologous antigen. The molecules forming the firmest linkages would be responsible for the neutralization of the virus, while the CF test would indicate also the very loose, dissociable bonds. In support of this view are the evidence obtained in our earlier immune serum absorption experiments [8], as well as the just described experimental data obtained in CF tests made in absorbed sera.

Summary

Influenza immune sera, untreated and absorbed with heterologous virus have been examined by the haemagglutination inhibition, the neutralization, the complement fixation and the complement fixation inhibition tests. The antibody contained in a given serum is indicated with variable titres by the different tests. The neutralization and the complement fixation inhibition tests afford the best differentiation between the homologous and the heterologous titres, while the haemagglutination inhibition test is less, and the complement fixation test is the least effective in this respect.

In studies on absorbed immune sera the neutralization and the complement fixation inhibition tests yielded results essentially identical with those obtained by the haemagglutination inhibition test. In the complement fixation test, however, even a serum completely absorbed with heterologous virus gave fixation with the same virus. In the light of the findings our hypothesis of the nature of antibody formed against the influenza virus has been discussed.

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ИССЛЕДОВАНИЕ РАЗЛИЧНЫМИ СЕРОЛОГИЧЕСКИМИ ПРОБАМИ СОДЕРЖАНИЯ АНТИТЕЛ В ИММУННЫХ СЫВОРОТКАХ, ИСТОЩЕННЫХ ВИРУСОМ ГРИППА

ДЬ. ТАКАЧИ и М. ХАМАР

Р е з ю м е

Авторы производили сравнительные исследования необработанных, а также истощённых гетерологичным вирусом гриппозных иммунных сывороток путем постановки реакций торможения гемагглютинации, нейтрализации, связывания комплемента и торможения связывания комплемента. Результаты различных проб не давали равнозначных титров антител в данных сыворотках. Гомологичный и гетерологичный титр наиболее дифференцируется в реакциях нейтрализации и торможения связывания комплемента, в более меньших размерах в реакции торможения гемагглютинации и наименее в реакции связывания комплемента. Результаты исследования иммунных сывороток, истощенных гетерологичным вирусом, в реакциях нейтрализации и торможения связывания комплемента принципиально были тождественны результату реакции торможения гемагглютинации. Именно, если после гетерологичного истощения в реакции торможения гемагглютинации гетерологичный титр исчезал, то в сыворотках не обнаруживался гетерологичный титр ни в реакции нейтрализации, ни с помощью реакции связывания комплемента. В противоположность этому в реакции связывания комплемента совершенно истощенная гетерологичным вирусом сыворотка связывается с истощающим вирусом. Однако — по мнению авторов — связывание это создается гомологичными антителами. Расхождения между результатами различных проб авторы объясняют тем, что молекулы антител связываются с антигеном с переменной силой и отдельные пробы показывают силу этого связывания.

A NEW E. COLI SEROTYPE, IDENTICAL IN ANTIGENIC STRUCTURE WITH TYPE 4b OF S. FLEXNERI

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Systematic investigations conducted during recent years have made it more and more obvious that the genera of Enterobacteriaceae are in an involved antigenic interrelationship. This statement applies to Shigellae, too. In an analysis of the antigenic relationships between Shigella and *E. coli*, EWING [1] has found among the 33 known serotypes of Shigella only 4 that exhibited no serological relationship to the *E. coli* group. In the majority of cases the antigenic relationship depends on partial antigens, but the O antigens of 10 Shigella types were found to be completely identical with *E. coli* group antigens. Four of the antigenically identical strains were in the subgroup A, 5 belonged to subgroup C, while the tenth strain exhibiting antigenic identity was *S. sonnei*.

To our knowledge, no strain of *E. coli* is known to be completely identical in antigenic structure with any member of subgroup B *(S. flexneri)*. In the serological relationships unmasked so far, the connective links between the *S. flexneri* and *E. coli* group antigens were represented mainly by the *S. flexneri* group antigens, but fractions of type antigens were also found in other instances. The relationship is markedly pronounced between the types 2a and 2b, respectively, and the strain designated 537—52 of the *E. coli* group O 13 [1]. The latter strain contains the whole type antigen of *S. flexneri* type 2a, but only a fraction of the group antigen. According to SEELIGER [2], a similarly close antigenic relationship exists between a typical *E. coli* strain isolated from a patient with dysentery and type 5 of *S. flexneri*. The coli strain contains the complete type antigen of *S. flexneri* type 5, but only a portion of the group antigen of that organism. The coli strain represents a new coli group designated O 129.

In the following, an *E. coli* strain, designated *E. coli pécs* is described, the antigenic structure of which is completely identical with that of *S. flexneri* type 4b. The strain merits attention also because it presents an interesting example of the variability of fermentation.

Results

The strain was isolated from the faeces of a patient exhibiting the clinical symptoms of dysentery. It grew in almost pure cultures on eosin-methylene-blue agar, and was composed of Gram-negative, non flagellated bacteria. By passing in U tube, however, cilia could be detected.

In the serum of the patient taken on the 14th day of illness, the strain agglutinated in a dilution as high as 1 : 320, which was recorded as a proof of pathogenicity.

The strain was identified as being *S. flexneri* by informative tests made in polyvalent Shigella sera. The cultural behaviour of growth in the polytropic medium used at our institute [3] also suggested Shigella. The strain agglutinated in sera III and IV* of the Flexner factor sera. On the basis of these findings

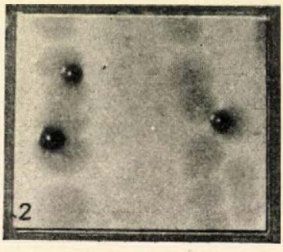

Fig. 1. Lactose fermenting papillae appearing on colonies not fermenting lactose. 24 hours incubation at 37 °C and 5 days at room temperature. Eosin-methylene blue medium

the strain was identified as *S. flexneri* type 4b. A detailed biochemical examination, however, has called attention to the fact that the strain may be considered to be a member of the *E. coli* group.

Cultural and biochemical properties. In indicator media containing lactose the strain formed colonies similar to those formed by bacteria not fermenting this carbohydrate. In 4 to 6 days at room temperature part of the colonies developed papillae fermenting lactose (Fig. 1).

Examination of colonies not forming papillae, from the papillae already mentioned, as well as from lactose-peptone water just after fermentation had taken place, has made it possible to study the variability of lactose fermentation.

From lactose-peptone water cultures spread on the 1st day of fermentation on eosin-methylene blue medium, beside typical *E. coli* colonies there have developed also colonies which did not ferment lactose (Fig. 2). The colonies decomposing lactose fermented it within 24 hours, and retained this property

* According to the terminology worked out by us [4], factor serum III is identical with MADSEN's group serum 6 [5], while serum IV is identical with MADSEN's factor serum IV.

also on prolonged passage in media not containing lactose. About 50 per cent of the colonies not utilizing lactose did not break down that carbohydrate in lactose-peptone water during the 14 days of observation, nor did they develop papillae; while the other 50 per cent formed regularly secondary colonies and exhibited late fermentation on re-inoculation into lactose-peptone water, indicating that lactose-utilizing variants were continually split off from them. The variants arising in secondary colonies broke down lactose rapidly, and also this property proved to be a persistent one.

The variability of gas production was also observed. On agar plates streaked with dextrose-peptone water cultures, incubated 5—6 days, 5 to 10 per cent of the colonies produced gas from all the fermentable carbohydrates and alcohols. On serial passages in dextrose broth the ratio of anaerogenic colonies gradually diminished and after 5 to 6 transfers there were exclusively gas

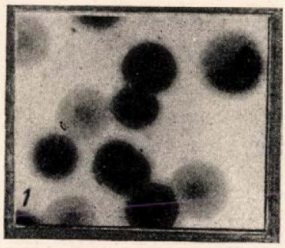

Fig. 2. Lactose fermenting and nonfermenting colonies from lactose peptone water, after 6 days of incubation. Eosin-methylene blue medium

producing colonies on the agar plates. As regards lactose fermentation, the gas producing variants showed the same variations as the anaerogenic variants.

The observations made on the variability of fermentation of lactose and gas production are illustrated in Fig. 3.

Table I shows the characteristic fermentative properties of the variants.

The rows in Table I show the following variants: rows 1, anaerogenic, row 4, aerogenic, without fermenting lactose; row 2, delayed lactose fermentation without gas production; row 5, delayed lactose fermentation with gas production; row 3, shows anaerogenic, but otherwise typically fermentating *E. coli* strain; row 6, *E. coli* strain behaving typically in all biochemical properties. As it has been pointed out, the delay in lactose fermentation finds its explanation in a gradual splitting off of lactose fermenting variants.

Antigenic structure. As already mentioned, the strain agglutinated in the polyvalent Flexner serum, as well as in the Flexner factor sera III and IV. Correspondingly, the strain was agglutinated up to the end titre by *S. flexneri* sera 1b, 3, 4a and 4b, and up to medium titre by the other sera. The strain

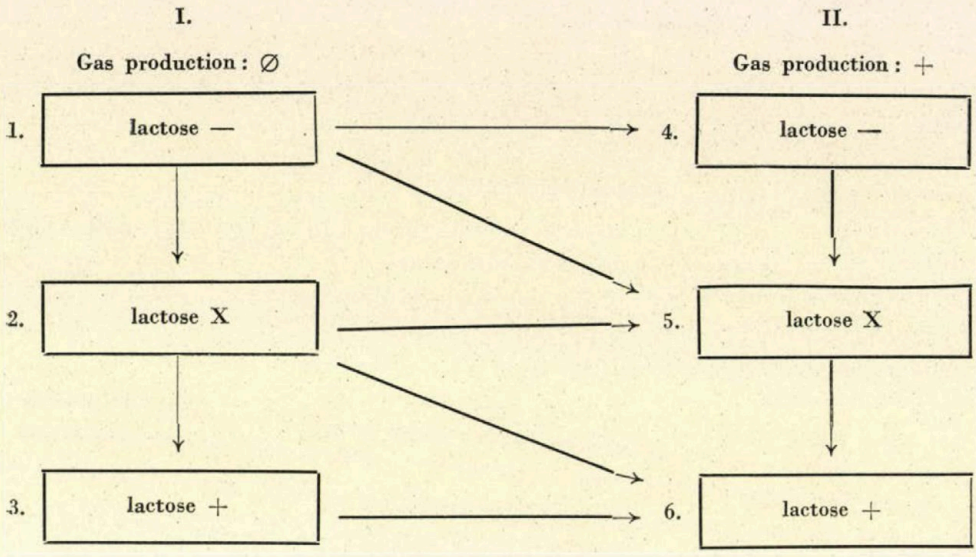

Fig. 3. The variability of *E. coli pécs*

was tested also in *E. coli* O sera 1 to 124 : it reacted markedly in serum O 13 and at low titres in sera O 17, O 19 and O 50. According to the control tests made by DR EWING (Communicable Disease Centre, Chamblee), and by DR. ØRSKOV (International Escherichia Centre, Copenhagen) — for which authors express

Table I

Biochemical reactions

Strains	Saccharose	Lactose	Dextrose	Mannit	Maltose	Xylose	Dulcit	Sorbit	Salicin	Adonit	Inosit	Urease	Indol	Methyl-red	Voges-Proskauer	Citrate	Gelatine
E. coli pécs var. 1....	—	—	+	+	+	+	—	+	+[5]	—	—	—	+	+	—	—	—
« « « « 2....	—	+[6]	+	+	+	+	—	+	+[5]	—	—	—	+	+	—	—	—
« « « « 3....	—	+	+	+	+	+	—	+	+[5]	—	—	—	+	+	—	—	—
« « « « 4....	—	—	⊕	⊕	⊕	⊕	—	⊕	⊕[5]	—	—	—	+	+	—	—	—
« « « « 5....	—	⊕[6]	⊕	⊕	⊕	⊕	—	⊕	⊕[5]	—	—	—	+	+	—	—	—
« « « « 6....	—	⊕	⊕	⊕	⊕	⊕	—	⊕	⊕[5]	—	—	—	+	+	—	—	—

\+ Acid production in 1 day, or positive ⊕ Acid and gas production in 1 day
+[6] Acid production in 6 days, or positive — Negative.

their thanks — there was no appreciable agglutination in the *E. coli* group sera 128 to 134, either. These control tests corroborated the existence of a relationship to *E. coli* O 13 antigen and demonstrated that the strain had minor relationships also to groups O 50, O 129 and O 133.

The characteristic data of the investigations into the antigenic structure of the strain have been tabulated (Table II).

Table II

Antigenic relationships

Strains (100[8])	O sera										
	E. coli pécs						S. flexneri 4b		E. coli 013		
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
	Before absorption	Absorbed					Unabsorbed	Absorbed	Unabsorbed	Absorbed	
		4b	3	4a «B»	Y	E. coli 013		E. coli pécs		E. coli pécs or S. flex. 4b	S. flex. var. Y
E. coli pécs	5120	—	1280	2560	5120	5120	10240	—	320	—	—
S. flexneri 4b	5120	—	1280	2560	5120	5120	10240	—	320	—	—
« « 3.....	5120	—	—	2560	5120	5120	5120	—	160	—	—
« « 4a «B»	2560	—	640	—	2560	2560	5120	—	320	—	—
« « var. Y.....	1280	—	—	—	—	—	1280	—	320	—	—
E. coli O13	1280	—	—	—	—	—	640	—	1280	1280	1280

According to the results of absorption tests, the strain has an identical antigenic structure with *S. flexneri* type 4b (III. IV). From this it follows that *E. coli pécs* serum is completely absorbed by the *S. flexneri 4b* strain (column 2) and that *S. flexneri 4b* serum is absorbed by *E. coli pécs*, without leaving behind any antibody (column 8).

The following experimental evidence lends further support to the validity of our findings.

1. The presence of antigen III is proved by the fact that, after having been absorbed with type 4a phase B, the *E. coli pécs* serum was still capable further to agglutinate strains containing antigen III (column 4).

2. The presence of antigen IV is indicated by the fact that the *E. coli pécs* serum absorbed with *S. flexneri* type 3 agglutinated the strains characterized by antigen IV (column 3).*

The relationship of the strain to the *E. coli* antigen O 13 is shown in columns 5, 6, 10 and 11 in Table II. According to these data, the relationship depends on partial antigens, because strain O 13 leaves the factors typical of *E. coli pécs* unaffected (column 6), and because the O 13 serum absorbed with

* Authors are indebted to DR. EWING for having corroborated this statement.

the pécs strain remains capable further to agglutinate the homologous strain (column 10). The connective link is apparently the common group antigen of the *S. flexneri* subgroup ; absorption with the Y variant (column 5), or any other Flexner strain will namely deprive the pécs serum from its ability to agglutinate the *E. coli* strain O 13 ; or, the relationship between the sera *E. coli* O 13 and pécs may be abolished by absorption with the Y variant or any of the Flexner types (column 11).

All fermentation types were identical in antigenic structure.

In connection with the characterization of the antigenic structure it has to be mentioned that the strain apparently contains no K antigen, as it was found that both the boiled and living suspension agglutinated equally well in antisera to living or boiled strain. The determination of H antigen was not made.

On the basis of these findings, and in agreement with DR. ØRSKOV, the strain may be considered to be *a representative of a new E. coli* O *group*. On the suggestion made by DR. ØRSKOV the strain was included in the Kauffmann-Knipschildt-Vahlne schema *as the type strain of the E. coli group* O 135.

Discussion

The strain *E. coli pécs* is, to our knowledge, the first *E. coli* serotype not distinguishable by serologic methods from a member (4b) of the *S. flexneri* subgroup. On the basis of present knowledge it could be expected that there would emerge an *E. coli* strain which is completely identical in antigenic structure with an *S. flexneri* type, just as it is certain that further *E. coli* seroanalogons will be discovered in future.

Research has explored but a small part of the dominion of enteric bacteria, thus it is only natural that new and new members of the various groups are and will be discovered. Although the strain *E. coli pécs* is in a definite serological relationship with the *E. coli* O 13 antigen, this depends on minor partial antigens and thus it is not justified to include the pécs strain into group O 13. The partial antigen referred to is common to most *S. flexneri* strains and is apparently identical with the group antigen connecting the Flexner strains. This observation corroborates the view put forward by SEELIGER that it is often the common group antigen of the *S. flexneri* types that is responsible for the cross reactions between *S. flexneri* and *E. coli* groups [6]. It can be assumed that the relationship demonstrated by EWING [1] between the *S. flexneri* types and the *E. coli* group O 13 depends upon the presence of the same factor (which, however, is certainly not identical with the antigens responsible for the relationship between *E. coli* 537—52 of group O 13 and *S. flexneri* 2a and 2b, respectively). Since the O antigen of the strain is not identical with those of the *E. coli* groups 1 to 134, it is justified to accept the strain as a

representative of a new coli O group, namely, O 135. The antigenic characteristic of coli group O 135 is that it is identical in antigenic structure with the O antigen of *S. flexneri* type 4b.

As regards fermentation, the *E. coli* strains containing Shigella antigens are often non-typical in behaviour [1]. The original strain of *E. coli pécs*, too, fermented non-typically and, in addition, its fermentative and gas producing properties were characterized by great lability. From the anaerogenic, lactose-negative variant an *E. coli* strain typical in its fermentative properties developed in a lactose milieu through a variant causing late fermentation of lactose. With the typically fermenting strain, the ability of decomposing lactose is a persistently established property. In the development from the non-typical form into the typical one the variant capable of causing late lactose breakdown occupies an intermediate position. The delayed lactose fermentation finds its explanation in a gradual development of variants capable of that action. These variants appear after a latency period in lactose media, and in solid media they form lactose positive papillae. In this state, strain *E. coli pécs* may be identified in cultural relations with the fermenting variant described by NEISSER and MASSINI [7, 8] under the name of *E. coli mutabile*. It would be interesting to examine the *E. coli* mutabile strains maintained in different laboratories for antigenic relationship to the pathogenic intestinal bacteria.

E. coli pécs has a variable gas producing ability. Gas production will take place in a milieu containing fermentable carbohydrate and in such an environment it has proved to be a progressing process, in the course of which all the originally anaerogenic variants assume a gas producing property.

The frequent occurrence of strains showing non-typical fermentation indicates that no sharp demarcation is possible on the basis of biochemical properties between the genera of Enterobacteriaceae. The antigenic relationships of biochemically defined genera, on the other hand, show that they are not demarcated sharply by antigenic characteristics either. These strains, aberrant from the systematic point of view, may be looked upon as being transitional forms from one group to the other. The transitions are very probably gradual. An example of this is the strain *E. coli pécs*, which antigenically is *S. flexneri*, while in fermentative properties it varies from the anaerogenic form not breaking down lactose to the form exhibiting fermentation typical of *E. coli*, with intermediate variants between the above two extremes. Further observations and careful study will undoubtedly contribute useful information concerning the phylogenetical significance of intermediate strains.

The pathogenicity of *E. coli pécs* stands proved. This fact may be recorded as a positive evidence in the still controversial problem of relationship of antigenic structure and pathogenicity [2].

As regards diagnosis, it can be stated that the discovery of the essential properties of the strain was the result of careful biochemical and serological

work. This may be considered as another evidence showing that the far-reaching biochemical and serological interrelations between enteric bacteria make a reliable diagnosis impossible, unless precise biochemical and serological examinations are carried out.

Summary

1. From the faeces of a patient showing typical clinical symptoms of dysentery a pathogenic strain of *E. coli* has been isolated. The antigenic structure of the strain proved to be identical with type 4b of *S. flexneri*.

2. Since the strain possesses an O antigen that is not identical with any of the known *E. coli* groups, it may be looked upon as being representative of a new *E. coli* O group, designated O 135.

3. As regards lactose fermentation and gas production, the strain showing fermentation typical of *E. coli* has gradually developed through intermediate forms from the originally isolated anaerogenic, lactose negative variant.

4. The finding has been analysed with respect to the interrelations and transitions between the genera of the family of *Enterobacteriaceae*. The phylogenetical and diagnostical significance of the finding has been discussed and the aspects relating to the relationship between antigenic structure and pathogenicity have been pointed out.

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НОВЫЙ СЕРОЛОГИЧЕСКИЙ ТИП E.COLI, СХОДНЫЙ ПО АНТИГЕННОЙ СТРУКТУРЕ С S. FLEXNERI ТИПА 4b

К. РАУШ и А. ВЕРТЕНЬИ

Резюме

1. Из стула больного с типичными симптомами дизентерии авторы выделили патогенный штамм E. coli, оказавшийся сходным по антигенной структуре с штаммом S. flexneri типа 4b.

2. Упомянутый штамм не мог быть отнесен ни к одной из известных групп E. coli ввиду различности О-антигена и поэтому может считаться представителем новой (0135) О- группы E. coli.

3. В отношении расщепления лактозы и образования газа, штамм из анаэрогенного, не разлагающего лактозу варианта переходил в обладающий стойкими свойствами и типичной для E. coli ферментативностью вариант путем постепенных переходов.

4. Находку анализировали в отношении интерреляций и переходов между биохимически определенными родами семейства Enterobacteriaceae, выдвинули ее значение с точки зрения эволюции и диагностики, а также указали на вопрос зависимости между антигенной структурой и патогенностью.

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VERSUCHE ZUR ISOLIERUNG EINES LITHOCHOLSÄURE ABBAUENDEN MIKROORGANISMUS. II

Von

G. WIX

Forschungsinstitut für Pharmazeutische Industrie, Budapest

(Eingegangen am 19. August 1955)

In einer früheren Arbeit [1] beschrieben wir die Isolierung von Lithocholsäure-abbauenden Mikroorganismen und einen Organismus, von dem wir festgestellt hatten, dass er eine bisher nicht beschriebene Species von Corynebacterium ist und dass er Steroiden von gewissen strukturellen Eigenschaften anzugreifen fähig ist. Aus den mitgeteilten Daten zogen wir den Schluss, dass der Organismus an mehreren Angriffspunkten, so auch von der Seitenkette her, den Abbau des Moleküls beginnen kann. In der vorliegenden Mitteilung berichten wir über Experimente, welche Aufschluss darüber gaben, welche Enzyme bei der Oxydation der Lithocholsäure fungieren und ob der in Frage kommende Organismus für selektiven Seitenkettenabbau anwendbar sei.

Methoden

Wir benützten die WARBURG-Methode. Die zu untersuchende Suspension wurde auf derselben Weise bereitet, wie wir es in unserer ersten Mitteilung beschrieben hatten. In den Hauptraum der Warburg-Gefässchen wurde je 1,0 ml von dieser Suspension eingemessen, sowie 1,4 ml mol/7,5 Phosphat-Puffer, das zu oxydierende Substrat in 0,2 ml Volumen, 0,2 ml dest. Wasser, bzw. andere Materiale, deren Wirkung wir untersuchen wollten. Das entstandene Kohlendioxyd wurde durch ein im mittleren kleinen Behälter untergebrachtes laugiges Filterpapier absorbiert. Die Wirkung der Blausäure untersuchten wir mit Hilfe der Kalzium-Zyanid-Methode von ROBBIE [2]. Die Temperatur des Wasserbades war 28° C. Die Sauerstoffverbrauchswerte bezieben sich auf die in der vorhergehenden Arbeit [1] berichteten Zellmengen. Wir gewannen die quantitativen Sauerstoffverbrauchswerte dadurch, dass wir die Substrate, die wir in die Seitengefässe eingemessen hatten, nach Abschluss der Manometer in den Hauptraum des Gefässes eingaben.

Ergebnisse und Besprechung

In der vorigen Mitteilung berichteten wir, dass Testosteron eines derjenigen Substrate ist, die die kleinste Sauerstoffverbrauchserhöhung verursachen. Die Oxydierbarkeit dieser Verbindung und der Lithocholsäure verglichen wir mit Hilfe der Azeton-Methode bei verschiedenen pH-Werten. Die Angaben in Abb. 1. zeigen, dass das pH-Optimum der Angreifbarkeit der zwei Verbindungen sich scharf unterscheidet. Es ist anzunehmen, dass in der Oxydation

dieser Verbindungen von verschiedener Struktur nicht alle Enzyme identisch sind. Die Aktivität dieser Enzyme scheint bereits durch einen so einfachen Eingriff, wie die Veränderung des pH-Wertes, beeinflussbar zu sein. Wir haben jedoch keinen solchen Wert gefunden, bei dem die Oxydierbarkeit der einen Verbindung im Verhältnis zur anderen vernachlässigt werden kann. Bei den benützten Versuchsanordnungen konnten wir also bei keinem der angenommenen Enzym-Systeme vollkommene oder beinahe vollkommene Hemmung erreichen.

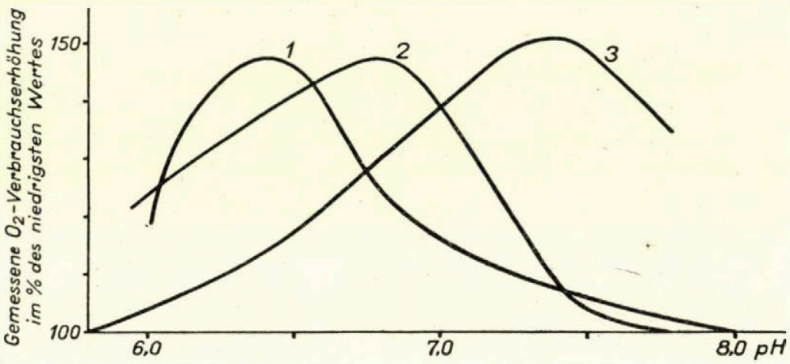

Abb. 1. Wirkung des pH auf die Steigerung der O_2-Aufnahme durch Testosteron bzw. durch Lithocholsäure. Alle Kurven sind die Resultanten von vier Versuchen. In jedem Versuch untersuchten wir die Wirkung des pH bei 5 verschiedenen Werten. Der Zusammenfassbarkeit willen legten wir die niedrigste O_2-Verbrauchssteigerung für 100, in jedem Experiment und bezogen die anderen Werte darauf

Danach untersuchten wir die Wirkung einiger Enzymgifte. Die erhaltenen Ergebnisse sind auf Abb. 2—8. dargestellt.

Wir benützten bei den Abbildungen einheitliche Bezeichnungen: Grundatmung: ·——·, Grundatmung + Inhibitor, oder anderes Material, dessen Wirkung untersucht wurde: - - - - - - -, lithocholsäurige Atmung: x—x—x, lithocholsäurige Atmung + Inhibitor: x— — —x, Menge der eingemessenen Lithocholsäure: 1,0 mg pro Gefäss. Name und Endkonzentration des Inhibitors wird unter jeder Abbildung angegeben.

Fluoressigsäure ist ein wohlbekanntes Hemmungsmittel des Zitronensäure-Zyklus. Ihre Wirkung wird durch Umwandlung in Fluorzitronensäure ausgeübt. Zur Entwicklung ihrer inhibitorischen Wirkung ist also eine gewisse Zeit nötig. Das ist in Abb. 2. gut veranschaulicht: der Inhibitor verhindert immer stärker den durch die Lithocholsäure verursachten Sauerstoffverbrauch. Gleichzeitig wirkt er auf die endogene Atmung leicht erhöhend. Lithocholsäure wird also durch den Zitronensäure-Zyklus metabolisiert, während dieser Zyklus in der endogenen Atmung des Organismus überhaupt keine, oder nur eine untergeordnete Rolle spielt. Die Rolle des Zitronensäure-Zyklus in der Oxydation der Lithocholsäure wird durch die in Abb. 3 mitgeteilte Beobachtung noch

verstärkt: Terramycin, von dem es bereits bekannt ist, dass es den Krebs-Zyklus verhindert, hemmt auch den oxydativen Abbau der Lithocholsäure.

Die Wirkung von Natriumazid als Inhibitor veranschaulicht Abb. 4.: Azid erhöht die endogene Atmung und hemmt hingegen die in Anwesenheit von Lithocholsäure gemessene Atmung. (Die Stimulations-Wirkung von NaN_3 ist aus der Literatur wohl bekannt: bei l-Phosphoglycerat-Dehidrogenase und Glukose-Oxydase Präparaten wurde ein solcher Effekt beschrieben [3, 4].

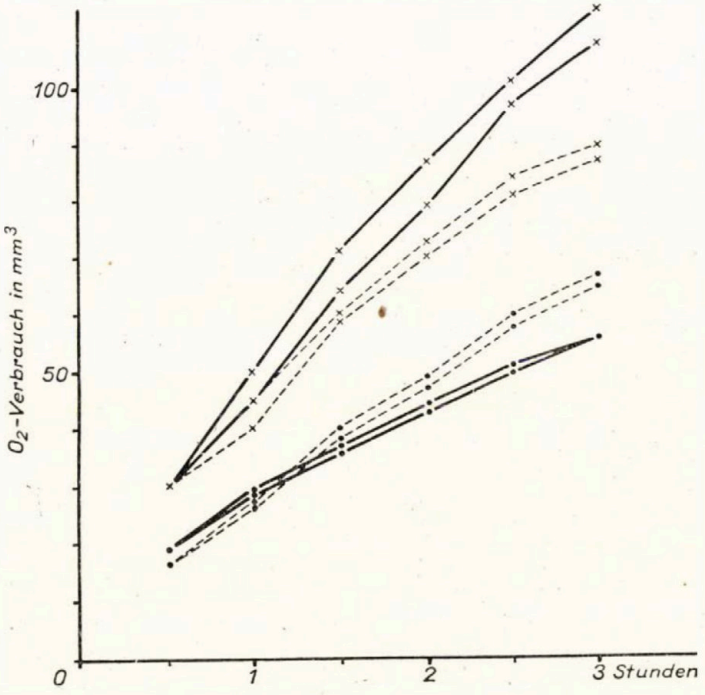

Abb. 2. Wirkung von 10 mM Fluoressigsäure bei pH 6·64

Gerade deswegen führten wir die Versuche in saueren pH-Intervallen durch, weil es bekannt ist, dass dieser Inhibitor z. B. d-Aminosäuren-Oxydase in schwach sauerem Medium hemmt, und bei pH 7,8 stimuliert [5]. Zwei Mechanismen können bei der Natriumazid-Hemmung in Frage kommen: Entweder ist die ATP-Synthese zurückgedrängt, oder die Tätigkeit der metallenthaltenden Atmungsfermente gehindert. Ein reines Bild zu gewinnen, untersuchten wir die Wirkung von Dinitrophenol (DNP), bzw. von Zyanid auf die in Frage kommende Reaktion. Ersteres erhöhte die endogene Atmung, verhinderte aber die der Lithocholsäure vollkommen; der in diesen Gefässen gemessene Sauerstoffverbrauch fiel in kurzer Zeit sogar unter den Endogen-Wert. Mit dieser Erscheinung befassen wir uns jetzt nicht weiter, nur wollen wir feststellen, dass beide Inhibitoren,

die die Synthese der energiereichen Phosphatester verhinderten, hemmend auf die Oxydation der Lithocholsäure wirkten. Die Zyanid-Versuche (Abb. 6.) zeugen jedoch davon, dass bei Verbrennung der Lithocholsäure auch metallenthaltende Atmungsfermente eine Rolle spielen, weil Zyanid die endogene Atmung kaum beeinflussend, auf die durch Lithocholsäure hervorgerufene Atmungserhöhung stark reduzierend wirkte.

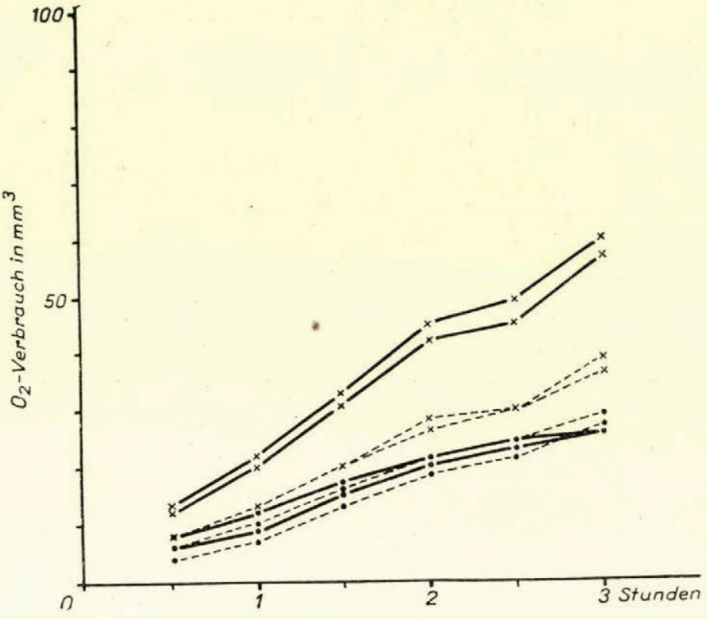

Abb. 3. Wirkung von 650 γ/ml Terramycin bei pH 6,64

Nun untersuchten wir die auf SH-Gruppen wirkende Inhibitoren. Monojodessigsäure (Abb. 7.) und Natriumarsenit (Abb. 8.) reagierten auf gleicher Weise: Beide verhinderten die endogene Atmung, sowie die Lithocholsäure-Atmung. In einigen Fällen war letztere Hemmung vielleicht stärker, doch sind die Angaben nicht genügend, um das als signifikant anzuerkennen.

Nachdem wir uns davon überzeugt hatten, dass die Oxydation der Lithocholsäure durch Enzymgifte beeinflussbar ist, untersuchten wir die Sauerstoffquantität, die bei der Oxydation des Lithocholsäure-Methyläthers verbraucht wird. Unsere Wahl fiel auf diese Verbindung, weil es aus früheren Experimenten bekannt war, dass sie eine bedeutend kleinere Erhöhung des Oxygenverbrauches verursacht als die Lithocholsäure. Wir nahmen an als Ursache der Erscheinung, dass der Organismus die Ätherbindung nicht angreifen kann und so das Molekül nur von der Seitenkette her abgebaut wird. Bei den Lithocholsäure-Methyl-

äther-Experimenten waren die Ergebnisse sehr verschieden. Die Ursache dieser Streuung suchend, dachten wir an das Waschen der Bakteriumsuspension, als auf einen Schritt, der auf exakte Weise schwer zu reproduzieren ist. So können bei jedem Experiment in verschiedener Quantität Nährbodenbestandteile in der Suspension zurückbleiben, und es besteht die Möglichkeit, dass diese auf die Oxydation des Substrates eine Wirkung ausüben. Um diese Ursache klar zu legen, haben wir statt dem üblichen, zweimaligen Waschen mit dest. Wasser,

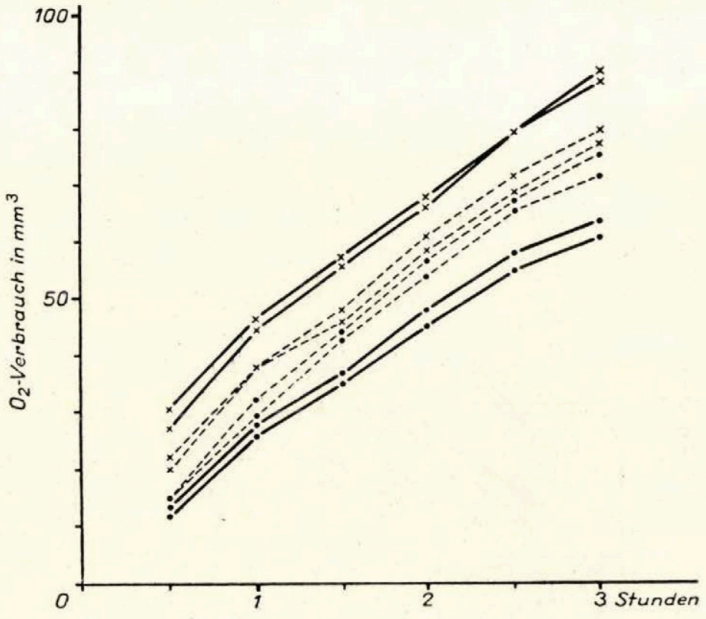

Abb. 4. Wirkung von 1 mM NaN_3 bei pH 6,24

dreimal gewaschen, und haben die Waschflüssigkeit nicht nur abgegossen, sondern das Sediment mit Filterpapier nach jedem Waschen getrocknet. Mit einer, auf diese Weise bereiteten Suspension experimentierend, untersuchten wir die Wirkung der verschiedenen Nährbodenbestandteile einzelweise und gemeinsam, sowie den Einfluss des ausgegorenen Mediums auf die Oxydation des Lithocholsäure-Methyläthers. (Zur Züchtung des Organismus bedienten wir uns des in der vorigen Mitteilung [1] beschriebenen und mit SA5 bezeichneten Mediums. Wir bereiteten von jedem der Bestandteile eine Lösung von solcher Konzentration, wie diejenige in dem Nährboden und dosierten von dieser Lösung je 0,2 ml in die Warburg-Gefässe.) Die Ergebnisse sind in Abb. 9. dargestellt: Zuckerfreier Nährboden SA5, Pepton- und Hefeextrakt waren effektlos, aber Fleischextrakt in der benützten Konzentration steigerte den durch Lithocholsäure-Methyläther verursachten Sauerstoffverbrauch. Das Ergebnis

des zuckerfreien Nährbodens SA5 wird dadurch erklärt, dass Hefeextrakt die Wirkung des Fleischextraktes annulliert. Hingegen wird der vorteilhafte Effekt des Fleischextraktes auf die Oxydation durch Pepton nur vermindert.

Hierauf untersuchten wir die Wirkung des ausgegorenen Nährbodens. Auch dieser erhöhte den durch Lithocholsäure-Methyläther ausgelösten Sauerstoffverbrauch.

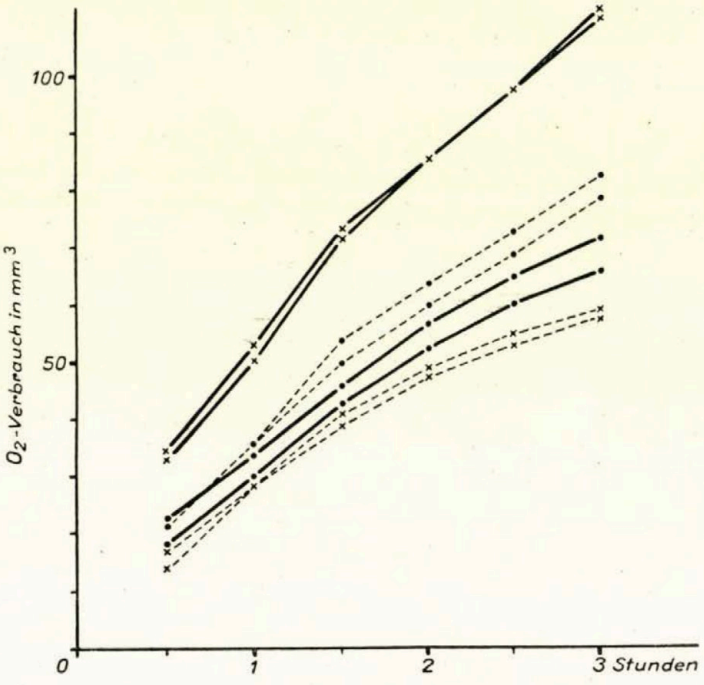

Abb. 5. Wirkung von 1 mM DNF bei pH 6,24

Nach dieser Feststellung arbeiteten wir nicht weiter mit gewaschener Zucht, sondern bereiteten mittels Zentrifugierung der Bakterien im ausgegorenen Nährboden eine konzentriertere Bakterien-Suspension: Nach der Zentrifugierung saugten wir die Hälfte der reinen Nährlösung ab und suspendierten die Bakterien im Rest. Von dieser Suspension gaben wir je 1,0 ml in Warburggefässe ein. Übrigens war die Versuchsanordnung dieselbe, wie die in der Methodik angegebene. Wir wogen in das Seitengefäss zweier Gefässe 0,2 mikromol Lithocholsäure ein, in das Seitengefäss zweier anderen 0,2 mikromol Lithocholsäure-Methyläther (beide in der Form von Natriumsalz). In die Seitengefässe der zwei letzten Gefässe setzten wir nur 0,2 ml dest. Wasser. Die Angaben der Tab. I gewannen wir dadurch, dass wir die in den zwei letzten Gefässen

bestimmte Sauerstoffverbrauchsmittelwerte aus den in den beiden ersten bzw zweiten Gefässen bestimmten Mittelwerten substrahierten.

Die Abweichung der Angaben erlaubt einen Sauerstoffverbrauch von rund 50 bzw. 100 mm^3 anzunehmen. Das bedeutet 10, bzw. 20 mol Sauerstoffverbrauch

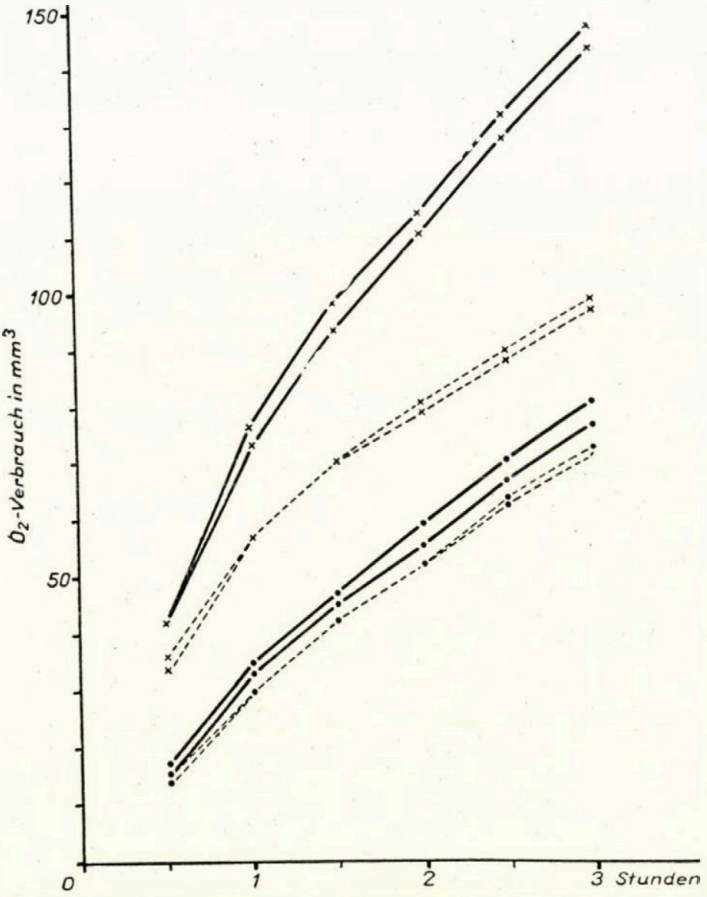

Abb. 6. Wirkung von 0,5 mM Zyanid bei pH 6,24

pro Substratmolekül. Wenn die Seitenkette gänzlich oxydiert wird so, dass auf der C—17 nur die Keto-Gruppe bleibt, ist der theoretische Sauerstoffverbrauch 7 mol. Der mit Lithocholsäure-Methyläther erreichte O_2-Verbrauch ist bedeutend grösser und man kann sich keine in diesem Grad oxydierte Struktur vorstellen, die noch zur Synthese von Steroid-Hormonen anwendbar wäre. Deswegen haben wir unsere Experimente mit diesem Stamm nicht fortgesetzt.

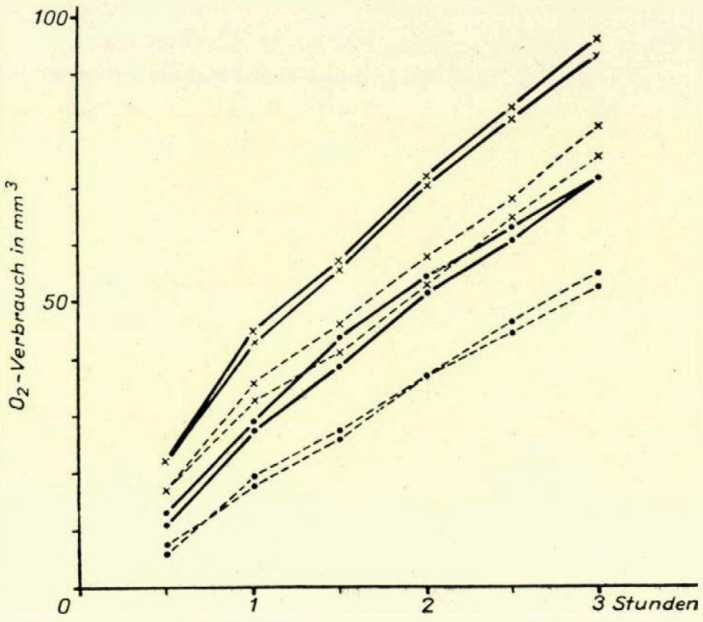

Abb. 7. Wirkung von 1 mM Na-Arsenit bei pH 6,24

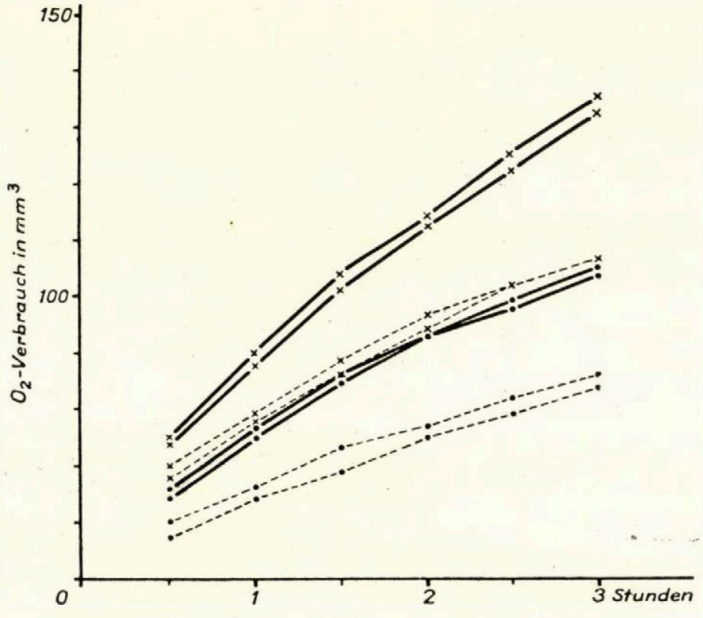

Abb. 8. Wirkung von 1 mM Monojodessigsäure bei pH 6,24

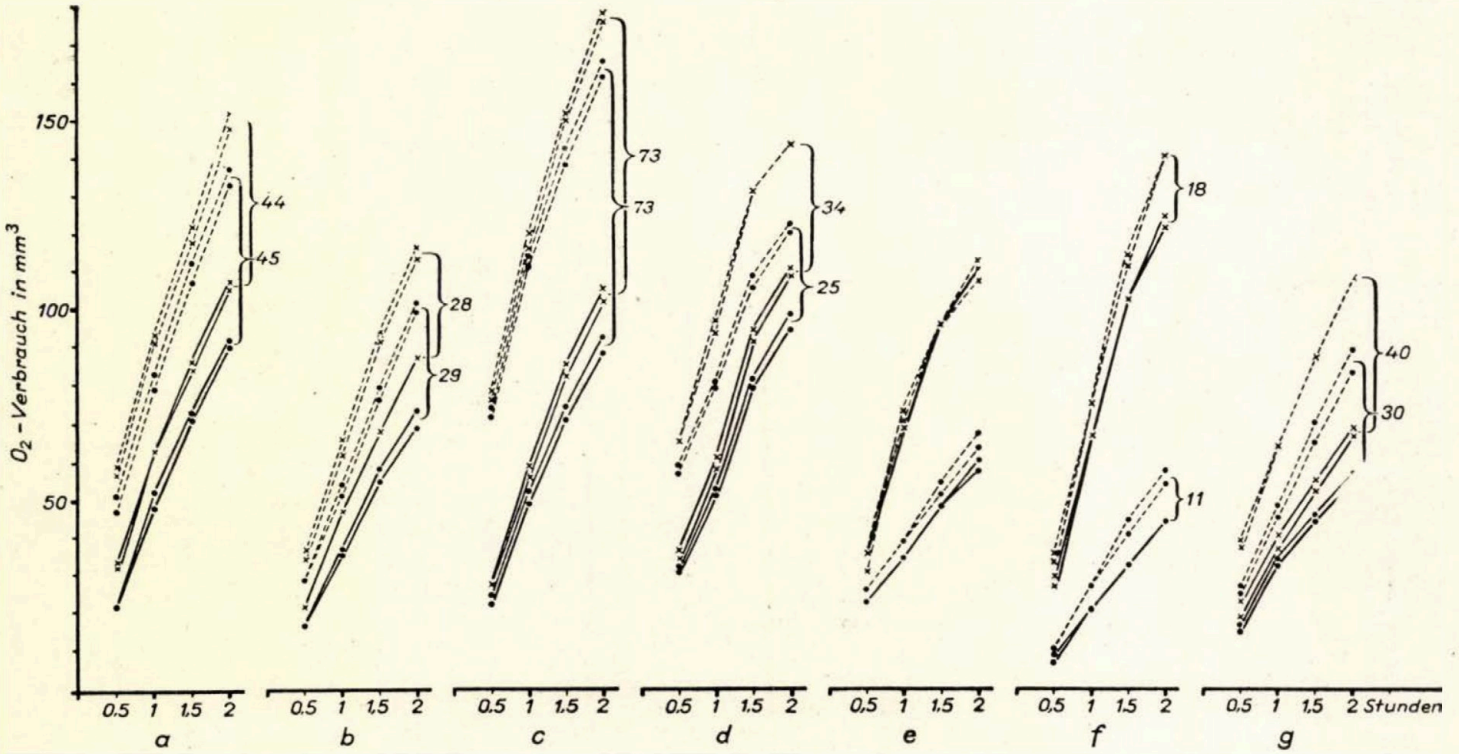

Abb. 9. Wirkung des *a)* zuckerfreien Nährbodens SA5, *b)* Peptons, *c)* Hefeextraktes, *d)* Fleischextraktes, *e)* Fleisch- und Hefeextraktes, *f)* Fleischextrates und Peptons, *g)* ausgegorenen Nährbodens auf den Sauerstoffverbrauch des Mikroorganismus

Tabelle I

Sauerstoffmenge verbraucht für die Oxydation von Lithocholsäure und Lithocholsäure-Methyläther

Datum	O_2-Verbrauch in mm^3 für 0,2 μ mol	
	Lithocholsäure-Methyläther	Lithocholsäure
16. 5. 1955	60	109
17. 5. 1955	49	100
18. 5. 1955	55	118
19. 5. 1955	49	95
20. 5. 1955	52	80
23. 5. 1955	56	100
24. 5. 1955	43	80
25. 5. 1955	49	116
26. 5. 1955	41	84
27. 5. 1955	40	80
30. 5. 1955	42	86
31. 5. 1955	59	101
Durchschnitt	49,6	95,7

Zusammenfassung

1. Das Lithocholsäure oxydierende System des für Lithocholsäure-Abbau isolierten Corynebacterium-Species kann durch die Hemmungsmittel des Zitronensäure-Zyklus (Fluoressigsäure und Terramycin), energiereiche Phosphatester Synthese beeinflussende Mittel (Azid und Dinitrophenol), sowie durch die Lähmungsmittel der Metall-Enzyme (Zyanid, Azid) gehindert werden. Über die Rolle der SH-Gruppen bekamen wir kein klares Bild.

2. Unter Umständen, wenn nur die Seitenkette angreifbar ist, ist der im Warburg-Versuch erreichte Sauerstoffverbrauch höher (10 mol pro Substratmolekül) als der theoretische Verbrauch bei dem vollständigen Seitenkettenabbau (7 mol pro Substratmolekül). Der Organismus ist also für den selektiven Abbau der Lithocholsäure-Seitenkette nicht geeignet.

Für ihre technische Hilfe gebührt Frau L. VIHAR mein besonderer Dank.

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ОПЫТЫ ПО ВЫДЕЛЕНИЮ МИКРООРГАНИЗМА, РАСЩЕПЛЯЮЩЕГО ЛИТОКОЛОВУЮ КИСЛОТУ. II

ДЬ. ВИКС

Р е з ю м е

Автор, исследуя описанный в предыдущем сообщении штамм, изолированный для расщепления литокола, установил следующее:

1. Окисляющую систему этого вида коринебактерии можно тормозить ингибиторами цикла лимонной кислоты, веществами влияющими на высокоэнергетический синтез фосфатного эфира, а также ингибиторами металлосодержащих энзимов. Точных данных о роли энзимов, содержащих группу SH, в этих опытах не было получено.

3. При таких условиях, когда имеется возможность поражения только боковой цепи, определенное в опыте Варбурга потребление O_2 оказалось большим (10 мол молекула субстрата), чем теоритический расход (7 молмолекула субстрата) при полном расщеплении боковой цепи. Следовательно данный организм не годен для селективного расщепления боковой цепи литоколовой кислоты.

NERVENSYSTEM UND IMMUNITÄT. VII

DIE WIRKUNG VON HYPOTHERMIE AUF DAS SHWARTZMAN-PHÄNOMEN

Von

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(Eingegangen am 20. September 1955)

In einer früheren Mitteilung [1] wurde darüber berichtet, dass es möglich ist, den an Meerschweinchen hervorgerufenen letalen anaphylaktischen Schock durch Hypothermie zu verhindern. Hierbei wurde festgestellt, dass sich die Histaminempfindlichkeit im hypothermischen Zustand nicht wesentlich ändert. Die Abwehrwirkung der Hypothermie gegenüber dem anaphylaktischen Schock wurde damit erklärt, dass der Ablauf der Antigen-Antikörper-Reaktion und die Histaminfreisetzung in den Tieren mit vermindertem Stoffwechsel stark abnehmen. In der vorliegenden Arbeit soll nun die Wirkung der Hypothermie auf das SHWARTZMAN-Phänomen untersucht werden.

Methodik

Als Versuchstiere wurden Kaninchen verwendet. Als sensibilisierende Injektion wurde 0,25 ml steriles Filtrat einer 48stündigen Agar- und Bouillonkultur von international standardisierten Coli dyspepsiae-Stämmen (Antigenstruktur O : 111, B 4; O : 55, B 5 und O : 26, B 6) intrakutan verabreicht. Als auslösende Injektion wurde 1 ml/kg Körpergewicht desselben Filtrats 24 Stunden nach der Erstinjektion intravenös gegeben. Nach 4 bis 5 Stunden hatte sich die Reaktion bereits vollständig ausgebildet. Die Unterkühlung wurde derart ausgeführt, dass die Tiere mit Gummisäcken umgeben wurden, die mit fein zerstückeltem und gesalzenem Eis gefüllt waren. Die Temperatur der Kaninchen sank nach 2 bis 3 Stunden unter die kritische Temperatur von 30 °C ; die Tiere wurden 4 bis 5 Stunden auf dieser Temperatur gehalten und danach in einem auf 30 °C eingestellten Thermostaten unterbracht, wo sie nach 2 bis 3 Stunden eine Körpertemperatur von 30 °C erreichten. *Eine pharmakotherapeutische Behandlung* (Anästhetika oder Narkotika) *gelangte in keinem einzigen Falle zur Anwendung.* Die Messung der Körpertemperatur erfolgte mit Hilfe eines Quecksilberthermometers, das 5 bis 6 cm tief in das Rektum eingeführt wurde.

Versuchsergebnisse

Zuerst wurde der Versuch an 10 Kontrollkaninchen ausgeführt ; an sämtlichen Tieren bildete sich das Shwartzman-Phänomen aus, wobei nekrotische Flecke von 2,5 bis 3,5 cm^2 bzw. hyperämische Ringe von 3 bis 4,5 cm^2 entstanden. Dann wurden 20 Kaninchen sensibilisiert, nach 24 Stunden auf die oben beschriebene Weise abgekühlt, worauf sie die Reinjektion im hypothermi-

schen Zustand erhielten. Wie aus Tabelle I hervorgeht, trat in jenem Falle, wo die Zweitinjektion einem Tiere über der kritischen Temperatur verabreicht wurde, das Shwartzmen-Phänomen, wenngleich in einem verminderten Ausmass (0,9 bis 1,5 cm^2 grosse Hyperämie mit punktartiger Nekrose), so dennoch auf. *Wurde jedoch die auslösende Injektion unterhalb der kritischen Temperatur verabreicht, so bildete sich das Shwartzman-Phänomen überhaupt nicht aus.* In diesen Fällen handelt es sich nicht um eine einfache Verzögerung der Reaktion, was auch dadurch zum Ausdruck kommt, dass selbst bei einer mehrwöchigen Kontrolle der Tiere keine Spuren der Symptome des Shwartzman-Phänomens wahrzunehmen waren.

Tabelle I

Auslösung des Shwartzman-Phänomens im hypothermischen Zustand

Kaninchen Nr.	Körpertemperatur bei der Auslösung	Ergebnis
1.	25 °C	negativ
2.	23 °C	negativ
3.	26 °C	negativ
4.	26 °C	negativ
5.	30 °C	Hyperämie: 0,9 cm^2 Nekrose: punktartig
6.	26 °C	negativ
7.	27 °C	negativ
8.	27 °C	negativ
9.	31 °C	Hyperämie: 1,2 cm^2 Nekrose: punktartig
10.	26 °C	negativ
11.	27 °C	negativ
12.	30 °C	Hyperämie: 1,4 cm^2 Nekrose: punktartig
13.	27 °C	negativ
14.	32 °C	Hyperämie: 1,5 cm^2 Nekrose: punktartig
15.	26 °C	negativ
16.	33 °C	Hyperämie: 1,3 cm^2 Nekrose: punktartig
17.	24 °C	negativ
18.	28 °C	negativ
19.	26 °C	negativ
20.	25 °C	negativ

Danach sollte die Frage geklärt werden, wie sich die Hypothermie auf die Ausbildung des Shwartzman-Phänomens auswirkt, wenn die Erstinjektion im hypothermischen Zustand gegeben wird. Laut der Versuche von STETSON [2]

besteht das Wesen des Shwartzman-Phänomens darin, dass die das Phänomen auslösenden Substanzen Thrombo- und Leukopenie verursachen. Aus diesem Grunde wurde bei den zur Klärung dieser angesetzten Versuchen auch die Leukozytenzahl der Kaninchen mit Aufmerksamkeit verfolgt, indem vor bzw. 4 Stunden nach der Erstinjektion die Zahl der Leukozyten bestimmt wurde. Die diesbezüglichen Ergebnisse sind in Tabelle II enthalten. Die Körpertemperatur der Tiere war vor der sensibilisierenden Injektion in jedem einzelnen Falle unter 30 °C. Bei 8 unter den 10 Kaninchen bildete sich das Shwartzman-Phänomen schwach aus (0,9 bis 1,6 cm^2 grosse Hyperämie, punktartige bis 0,5 cm^2 Nekrose), während es in 2 Fällen vollständig ausblieb. Die Leukozytenzahl sank nach der auslösenden Injektion in jedem Fall beträchtlich, ausgenommen bei den zwei Kaninchen, bei denen sich das Phänomen überhaupt nicht ausbildete.

Tabelle II

Untersuchung des Shwartzman-Phänomens: Erstinjektion in Hypothermie, Zweitinjektion in Normothermie

Kaninchen Nr.	Körpertemperatur bei Gabe der Erstinjektion	Ergebnis	Leukozytenzahl vor der Zweitinjektion	Leukozytenzahl 4 Stunden nach der Zweitinjektion
21.	26 °C	Hyperämie: 1,6 cm^2 Nekrose: punktartig	7400	3900
22.	25 °C	Hyperämie: 1,4 cm^2 Nekrose: punktartig	8500	6000
23.	24 °C	Hyperämie: 1,5 cm^2 Nekrose: 0,5 cm^2	9200	3300
24.	29 °C	Hyperämie: 1,2 cm^2 Nekrose: punktartig	8400	3800
25.	26 °C	Hyperämie: 1,0 cm^2 Nekrose: punktartig	5300	2100
26.	24 °C	Hyperämie: 1,6 cm^2 Nekrose: 0,5 cm^2	12000	5700
27.	26 °C	negativ	9300	9800
28.	27 °C	Hyperämie: 1,4 cm^2 Nekrose: 0,3 cm^2	10000	4000
29.	29 °C	Hyperämie: 0,9 cm^2 Nekrose: 0,4 cm^2	6700	3000
30.	25 °C	negativ	6000	5300

Diskussion

Das Ausbleiben des anaphylaktischen Schocks in der Hypothermie wurde mit einer starken Verminderung des Stoffwechsels erklärt und unserer Meinung nach lässt sich das Ausbleiben des Shwartzman-Phänomens, obgleich zwischen

diesen beiden Erscheinungen wesentliche Unterschiede bestehen, ebenfalls auf die Verminderung des Stoffwechsels zurückführen. Wenn die auslösende Injektion im hypothermischen Zustand verabreicht wird, tritt das Phänomen überhaupt nicht auf, wird dagegen die sensibilisierende Injektion im hypothermischen Zustand gegeben, so tritt es entweder nur in schwacher Form in Erscheinung oder bleibt vollständig aus. Laut unserer Versuchsergebnisse ist also nicht nur *zur Auslösung der hämorrhagischen lokalen Reaktion, sondern auch zu ihrer Vorbereitung, zur Bindung des Wirkstoffes eine bestimmte Intensität des Stoffwechsels notwendig.* In diesem Zusammenhang ist es beachtenswert, dass das Shwartzman-Phänomen an kaltblütigen Tieren nicht hervorgerufen werden kann. Von Bedeutung ist auch, dass sich im Falle der negativen Reaktionen der zweiten Versuchsreihe die Zahl der Leukozyten nicht wesentlich geändert hatte. Aus dieser Beobachtung sollen jedoch keine weiteren Folgerungen auf den Mechanismus und die Ursache des Shwartzman-Phänomens gezogen werden, zumal die diesbezügliche Rolle der Leukozyten in der letzten Zeit stark umstritten ist [3].

KESZTYÜS und Mitarbeiter [3] befassten sich eingehend mit der Rolle des Nervensystems im Mechanismus des Shwartzman-Phänomens, wobei sie die Entstehung dieses Phänomens nach Durchschneidung des N. ischiadicus, auf einem nach MANSFELD denervierten Hautlappen und in Sevenalnarkose untersuchten. Sie stellten hierbei fest, dass nur die Narkose eine relativ hemmende Wirkung ausübt. Laut ihrer Meinung gelangt der Einfluss des Zentralnervensystems nicht über die peripheren Nerven, sondern eher auf hormonalem-humoralem Wege zur Geltung. Nach den Versuchen von JENTZER [4] tritt das Shwartzman-Phänomen an hibernisierten Kaninchen nicht auf. Es stellt sich nun die Frage, was für ein Zusammenhang und was für eine Beziehung zwischen dem Nervensystem und der Hypothermie besteht. Da die Erregung des Nervensystems nichts anderes als eine infolge der Reize eintretende Stoffwechselveränderung ist, zieht die starke Verminderung des Stoffwechsels auch eine Verminderung der Aktivität des Nervensystems nach sich. Andererseits wird der Stoffwechsel durch das Nervensystem den Veränderungen des Aussen- und Innenmilieus gemäss so reguliert, dass die Adaptation des Organismus so vollkommen wie nur möglich gewährleistet wird. Durch die auf das Nervensystem ausgeübte Wirkung kann also auch der Stoffwechsel verändert werden. Auf Grund dieser Tatsachen ist es offenbar, dass das Shwartzman-Phänomen im hypothermischen Zustand deswegen ausbleibt, weil einerseits der Stoffwechsel stark sinkt und andererseits — infolge der Stoffwechselverminderung — die neurohumorale Regulation, die bei der Entstehung des Shwartzman-Phänomens eine Rolle spielt, schwer gestört wird.

Zusammenfassung

Das SHWARTZMAN-Phänomen kann durch Hypothermie vollständig verhindert werden, wenn die auslösende Injektion im hypothermischen Zustand verabreicht wird. Die Symptome dieses Phänomens bilden sich weit schwächer aus oder bleiben eventuell völlig aus, wenn die sensibilisierende Injektion dem Versuchstier im hypothermischen Zustand gegeben wird. Die Ursache für das Ausbleiben der Erscheinung liegt in der starken Stoffwechselverminderung und in der dadurch hervorgerufenen Störung der neurohumoralen Regulation.

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НЕРВНАЯ СИСТЕМА И ИММУНИТЕТ. VII

Влияние гипотермии на феномен Шварцмана

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Резюме

Феномен Шварцмана может быть полностью предотвращен гипотермией, если в состоянии гипотермии введем разрешающую инъекцию. Симптомы феномена в большой степени понижаются, иногда и совсем отсутствуют, если в состоянии гипотермии введем препаровальную инъекцию. Причиной отсутствия феномена является значительное понижение обмена веществ, и наступающее вследствие этого расстройство нейрогуморальной регуляции.

VERSUCHE ZUR ISOLIERUNG EINES LITHOCHOLSÄURE ABBAUENDEN MIKROORGANISMUS. III

OXYDATION VON STEROIDEN DURCH TRICHODERMA VIRIDE

Von

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In unserer Mitteilung [4] wurde darüber berichtet, dass aus Bodenmuster Mikroorganismen isoliert werden konnten, welche in Warburg-Versuchen auf Zugabe von Lithocholsäure mit Erhöhen des Sauerstoffverbrauches reagierten. In derselben und in der folgenden Mitteilung [5] wurden die Resultate von Versuchen mitgeteilt, in welchen die Lithocholsäure oxydierende Fähigkeit eines von uns isolierten Bakteriums — eine Art Corynebacterium — untersucht wurde. Hier wollen wir nun unsere Versuche mit einem anderen isolierten Mikroorganismus — Stamm FA3—1 — beschreiben.

Methoden

Der Stamm FA3—1 wurde auf dem folgenden Nährboden gezüchtet.

Lösung A: 20 g Glukose
1 g NH_4NO_3
0,25 g K_2HPO_4
0,25 g $MgSO_4 \cdot 7H_2O$
5 mg NaCl
333 mg Fleischextrakt-Trockenstoff
mit dest. Wasser auf 1 l ergänzt

Lösung B: 485 mg $FeSO_4 \cdot 7H_2O$
14 mg $CaCl_2$
11 mg H_3BO_3
5 mg $Co(NO_3)_2 \cdot 6H_2O$
0,4 mg $CuSO_4 \cdot 5H_2O$
0,04 mg $MnCl_2 \cdot 4H_2O$
1,8 mg $(NH_4)_6Mo_7O_{27} \cdot 4H_2O$
8,5 mg $ZnSO_4 \cdot 7H_2O$
mit dest. Wasser auf 1 l ergänzt

Aus Nährlösung A wurden 100—100 ml in 500 ml Erlenmeyer-Kolben gegeben und 15 Minuten lang bei 120 °C im Autoklaven sterilisiert. Nach dem Auskühlen wurde zu jedem Kolben je 0,5 ml der vorher sterilisierten Lösung B gegeben. Dieser Nährboden unterschied sich von dem bei der Isolierung des Stammes gebrauchten nur darin, dass dieser als Kohlenstoff-Quelle 2% Glukose, während jener Formyllitocholsäure enthielt. Der Nährboden wurde mit je 1 ml einer von 7—14 Tage alter Kartoffelschrägagar-Kultur mit 5 ml dest. Wasser abgewaschenen Sporen-Suspension des FA3—1 Stammes geimpft. Die Kolben wurden auf einen, in einem Thermostat-Zimmer von 28 °C befindlichen Rütteltisch, mit einer Umdrehungszahl von 100 und 2,5 cm Schwingungsamplitude gestellt. Zu den Warburg-Versuchen wurden 1—4 Tage alte Kulturen verwendet, nachdem sie zentrifugiert wurden, und der Niederschlag mit sterilisiertem dest. Wasser 2mal gewaschen und in einem weder Kohlenstoff-Quelle noch Fleischextrakt enthaltenden Nährboden suspendiert wurde. Aus dieser Suspension wurden in 4 Warburg-Gefässe je

4,5 ml gegeben, dann zu 2 der Gefässe 0,2 ml dest. Wasser, zu den zwei anderen 0,2 ml einer 1 mg pro ml Formyllitocholsäure-Na (FLS) enthaltenden Lösung. In den kleinen Behälter der Warburg-Gefässchen wurde ein Stückchen Filtrierpapier gelegt und 0,3 ml 20%-ige NaOH-Lösung hinzugegeben, um das entstehende Kohlendioxyd zu absorbieren. Die Temperatur des Wasserbades war 28 °C.

Zu den präparativen Versuchen wurde ein 1% Glukose und 2% »corn steep liquor« enthaltender Nährboden verwendet, dessen Handhabung sich als leichter bewies ; das pH wurde auf 6,0 eingestellt. Sterilisierung und Beimpfung erfolgten in der oben beschriebenen Weise.

Versuchsergebnisse und Besprechung

Die Ergebnisse der Warburg-Versuche sind in der Tabelle zusammengefasst. Die Daten bedeuten den Mittelwert des Sauerstoffverbrauches in mm^3 während 4 Stunden in 2—2 parallelen Kolben.

Tabelle

Oxygen-Verbrauch des Stammes FA3—1 in Gegenwart von FLS und in seiner Abwesenheit

1. Grundatmung	2. Atmung in Anwesenheit von FLS	3. 2 ausgedrückt in % von 1
620	990	160
455	518	114
598	658	110
720	800	111
605	914	151

Es ist zu sehen, dass sich die Sauerstoffaufnahme in Gegenwart von FLS erhöht. Wird statt FLS Lithocholsäure angewandt, werden ähnliche Ergebnisse erhalten. Zur Bestätigung dessen, dass die Erhöhung des Sauerstoffverbrauches die Oxydation der Lithocholsäure durch den Organismus bedeutet, wurden zu insgesamt 2 l einer 24 Stunden alten Schüttel-Kultur 450 mg Lithocholsäure-Na in wässeriger Lösung gegeben und dann 3 Tage lang geschüttelt. Das Fermentat wurde filtriert, das Myzel 2mal mit 100 ml Azeton, dann 2mal mit 100 ml Chloroform gewaschen und die Wasch-Flüssigkeiten mit dem Filtrat vereinigt. Die vereinigten Flüssigkeiten wurden auf pH 4 eingestellt und 1 Tag stehen gelassen. Während dem Stehen schied sich ein lockerer brauner Niederschlag aus, welcher filtriert wurde. Das Filtrat wurde mit 3×1 l Chloroform extrahiert, der Extrakt mit Wasser gewaschen und über Magnesiumsulfat getrocknet. Nach Eindampfen in Vakuum wurden 0,56 g braunes Öl gewonnen, aus welchem nach Zugabe von wenig Chloroform 0,08 g weisse Kristalle erhalten wurden mit einem Schmp. [175]—183—184 °C. Zugabe von Lithocholsäure führte zu keiner Depression. Die Mutterlaugen wurden in einem Glykolmonophenyläther-Hexan System papierchromatograpisch untersucht [3] mit Phosphormolybdänsäure entwickelt ; es wurden doch nur verwaschene, nicht identifizierbare Flecken erhalten.

Da diese Versuche keine Aufklärung über die im Steroidmolekül durch dem Mikroorganismus verursachte Umwandlung gaben, wurden um die Frage

zu klären im Warburg-Versuch verschiedene Substrate angewandt. Als jedoch die bei der in unserem ersten Aufsatz beschriebenen sogenannten Azeton-Methode benützten Azeton, Alkohole oder Propylenglycol als Lösungsmittel verwendet wurden, wurde die von der Lithocholsäure verursachte Erhöhung des Sauerstoffverbrauches von der durch die Lösungsmittel verursachten Erhöhung verdeckt.

Bei den folgenden präparativen Versuchen diente Progesteron als Substrat. Zu 4 l einer 24 stündigen, gelüfteten, gerührten FA3—1 Kultur wurde unter sterilen Verhältnissen 1 g Progesteron in 25 ml Azeton gelöst zugefügt. Es wurde weitere 3 Tage fermentiert, dann das Myzel filtriert, mit 4×100 ml Azeton, dann mit 4×100 ml Chloroform gewaschen. Die Waschflüssigkeit wurde mit dem Filtrat vereinigt, dann die gesamte Flüssigkeit mit 3×2 l Chloroform extrahiert. Die Chloroform-Extrakte wurden vereinigt, mit 2×500 ml Wasser gewaschen, über Magnesiumsulfat getrocknet, dann in Vakuum eingeengt. Der Rückstand, ein braunes Öl wog 1,98 g. Nach Zugabe von 5 ml Äther schieden sich Kristalle ab, welche filtriert wurden: 0,35 g, Schmp. 120—122 °C. Die Kristalle wurden papierchromatographisch untersucht, in Propylenglykol-toluol und Carbitol-methylcyclohexan Systemen nach ZAFFARONI [6] mit Phthalsaurem-p-phenylendiamin besprüht. [1] Die Untersuchungen zeigten die Gegenwart eines, mit dem 11α-Oxyprogesteron identisch laufenden Stoffes. Die Mutterlauge wurde in 5 ml abs. Benzol aufgenommen und über 50 g BROCKMANNschem Aluminiumoxyd chromatographiert. Aus den Benzol und Benzol-5%Äther enthaltenden Fraktionen konnte 0,11 g Progesteron (Schmp. 125—127 °C) zurückgewonnen werden. Aus den Äther-5% Chloroform Fraktionen wurde 0,02 g 11α-Oxyprogesteron kristallinisch isoliert (Schm. 153—161 °C). Dieser Stoff gab mit authentischem 11α-Oxyprogesteron keine Schmelzpunkt-Depression. Die anderen Fraktionen enthielten nicht kristallisierende Öle, welche aus Gemischen bestanden: sie enthielten 11α-Oxyprogesteron und einen bisher nicht identifizierten, stärker polaren Stoff.

Es konnte angenommen werden, dass der stärker polare Stoff sich aus dem 11α-Oxyprogesteron bildete. Deshalb wurde bei identischen Fermentationsumständen 11α-Oxyprogesteron als Substrat angewandt und untersucht, ob sich der stärker polare Stoff bildet. Bei den Versuchen konnte jedoch das 11α-Oxyprogesteron unverändert zurückgewonnen und kein stärker polarer Stoff nachgewiesen werden. So bildet sich der stärker polare Stoff nicht aus 11α-Oxyprogesteron.

Die mikrobiologische Oxydation des Progesterons zu 11α-Oxyprogesteron ist ein wichtiger Teil der Cortison-Synthese [2]. Deswegen war die Identifizierung dieses Stammes — dessen Fähigkeit Steroide zu oxydieren bisher unbekannt war — von Interesse. Folgende Eigenschaften des Stammes wurden festgestellt: auf Kartoffelagar-Platte bildet sich ein schnell verbreitendes, zuerst weisses, dann hellgrün werdendes wolliges Gewächs. Sterile Lufthyphen können auch bei

älteren Kulturen oft beobachtet werden. Von der Unterseite betrachtet, erscheint die Kultur dunkelbraun ; die Agarplatte ist vom Pilz braun pigmentiert. Der Mikroorganismus wächst gut auf verschiedenen Nährböden. In der »slide cell« können im Alter von 48 Stunden nahe zum Rand des Agars grüne Fruchtkörper -Aggregate mit freiem Auge beobachtet werden. Bei der mikroskopischen Untersuchung sind verzweigende, septierte Fäden sichtbar, an denen die Conidiophoren sitzen, auf deren charakteristisch verdünnten Spitzen in runden Haufen von einem Durchmesser von cca 10 μ die Sporen (Durchmesser 2—2,5 μ) Platz nehmen. Der Stamm wird auf Grund dieser Befunde als *Trichoderma viride* betrachtet.

Für die technische Hilfe danken wir hier Frau L. VIHAR, und Frl. I. ANDRÁSSY.

Zusammenfassung

1. Aus Bodenmustern wurde ein Mikroorganismus isoliert, welcher — den Daten der Warburg-Versuche gemäss — Lithocholsäure zu oxydieren imstande ist.

2. Es konnte nicht festgestellt werden welche Verbindungen bei der Oxydation der Lithocholsäure durch den Mikroorganismus entstehen.

3. Der Mikroorganismus oxydiert das Progesteron zu 11α-Oxyprogesteron und einem stärker polaren Stoff.

4. Der Mikroorganismus wurde als *Trichoderma viride* identifiziert.

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ОПЫТЫ ПО ВЫДЕЛЕНИЮ ОРГАНИЗМА, РАСЩЕПЛЯЮЩЕГО ЛИТОХОЛОВУЮ КИСЛОТУ. III

Окисление стероидов с Trichoderma viride

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Резюме

Авторы изолировали из почвы организм (4), который — по данным опыта Варбурга — оксидировал литохоловую кислоту. Какое соединение образовалось из литохоловой кислоты установить не удалось. Организм оксидировал прогестерон в 11-α-оксипрогестерон и еще в более полярное вещество. Организм авторы находят идентичным Trichoderma viride.

UNTER DEM EINFLUSS DER CORTISONBEHANDLUNG ENTSTEHENDE LUNGENASPERGILLOSE BEI RATTEN

Von

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Cortison und ACTH finden immer mehr und mehr klinische Anwendung. Neben der wertvollen therapeutischen Wirkung dieser Mittel weisen viele klinische und Versuchsangaben darauf hin, dass ihre chronische Verabreichung die Widerstandsfähigkeit des Organismus verschiedenen Infektionen gegenüber nachteilig beeinflusst. Als Ursache der sich während der Cortisonbehandlung entwickelnden Infektionen werden im allgemeinen die antiphlogistische Wirkung des Medikamentes bezeichnet. Zweifellos erhöhen die Gefässpermeabilität hemmende Wirkung, die Unterdrückung bzw. Verlangsamung der entzündlichen Reaktion, die Leukozytenauswanderung und die Verminderung der Fibrinogenese die Empfindlichkeit Infektionen gegenüber und dadurch auch die Möglichkeit einer Vermehrung der Krankheitserreger (KASS, GENNAN, FINLAND [7]). Ferner wurde nachgewiesen, dass Cortison die Antikörperbildung stark hemmt. ANTOPOL [1] z. B. beobachtete bei Tieren in Immunitätsversuchen gegen das O-Antigen der Salmonellagruppe, dass unmittelbar nach der Entwicklung der vollständigen Immunität der Antikörpertiter des Serums bereits durch eine einzige höhere Dosis Cortison sogar um 50% vermindert wurde.

Neuerdings befasste sich eine Forschergruppe mit der Pilzinfektion fördernden Rolle des Cortisons. FRIEDMANN und Mitarbeiter [6] beobachteten bei Mäusen unter dem Einfluss von kombinierter Cortison-Verabreichung und Röntgenbestrahlung die Dissemination der intraperitoneal injizierten *Blastomyces dermatitidis*- und *Candida albicans*-Kulturen. CAVALLERO und SALA [3] bewerteten die Cortisonwirkung bei experimenteller Coccidiomykose. Bei ihren Versuchen gingen die behandelten Tiere an diffusen Milz- und Lungenläsionen zugrunde, während bei den Kontrolltieren lediglich an der Behandlungsstelle lokale Granulomen entstanden. FARREL und Mitarbeitern [5] gelang es bei Hunden, KÖNIGSBAUER [8] bei Ratten, mittels Cortison generalisierte Histoplasmose zustande zu bringen. SELIGMAN [11] führte mit verschiedenen Antibiotika, ferner mit Cortison systematische Candidiasis (Moniliasis) herbei.

Andere Forscher (NEWCOMER, WRIGHT [10]) beobachteten nur eine mässige Verschlimmerung der Pilzinfektion unter dem Einfluss von Cortison, andere (BAUM, SALVADOR [2]) wiederum schrieben sogar eine fungistatische

Wirkung dem Cortison zu. In diesem Zusammenhang erwähnte DE LAMATER [4], dass die Empfindlichkeit einzelner Tierarten Cortison gegenüber veränderlich ist, und stellte fest, dass die Resistenz der Mäuse gegen Infektionen nur durch sehr grosse Dosen vermindert werden kann.

In den gegenwärtigen Experimenten wurde die Wirkung des Cortisons auf die überimpfbaren Tumoren von Mäusen und Ratten untersucht. Die Untersuchungen wurden in drei Serien durchgeführt.

Serie I. 41 Mäuse mit Crocker-180-Sarkom erhielten durchschnittlich 38 Tage hindurch täglich 0,5 mg Cortison.

Serie II. 40 Ratten mit M-1-Sarkom wurden durchschnittlich 31mal täglich mit 2 mg Cortison behandelt.

Serie III. 23 Ratten mit Guérin-Karzinom wurden je 5 mg Cortison durchschnittlich 12mal verabreicht.

Das Cortison wurde bei jeder Serie mit 0,2 ml physiologischer Kochsalzlösung verdünnt, intraperitoneal injiziert. Die Kontrolltiere erhielten unter ähnlichen Umständen nur 0,2 ml physiologische Kochsalzlösung.

Die Behandlung wurde im allgemeinen bis zur spontanen Verendung der Tiere fortgesetzt. Über die Wirkung auf die Tumoren und die Metastasenbildung wird an einer anderen Stelle berichtet [9]. Hier werden wir uns nur mit der Frage der während der Behandlung auftretenden infektiösen Prozesse beschäftigen.

Bei der Tötung bzw. spontanen Verendung der Versuchstiere wurden bei Serie II in acht Fällen, bei Serie III in zwölf Fällen in den Lungen makroskopisch verstreute, gräulichweisse Knoten von der Grösse farbiger Stecknadelknöpfe oder Pfefferkörner beobachtet, die als Metastasen betrachtet wurden. Histologisch erwiesen sich diese Knoten meistens als mykotische, seltener als bakterielle Abszesse. Bei Serie II. wurden sechs bakterielle, zwei mykotische, bei Serie III elf mykotische und ein bakterieller Abszess gefunden. Die Pilze bestanden aus Myzelien und bildeten Kolonien wechselnder Grösse. Zwecks Identifizierung wurden die Schnitte nach MACMANUS—HOTCHKISS gefärbt und in einigen Fällen wurde auch eine Abimpfung vorgenommen (Abb. 1).* Bei Mäusen wurde nur in einem Falle Aspergillose gefunden, bakterielle Infektion dagegen in keinem Fall.

Unter dem Einfluss des Cortison traten infektiöse Lungenprozesse bei den Ratten auf. Auf Wirkung von 5-mg-Dosen entwickelte sich bei 56,5% der Tiere Lungenaspergillose. Bei den unbehandelten tumorösen Tieren kam keine Lungenmykose vor, die Tumorkachexie spielt also bei der Entwicklung der Mykose keine Rolle. Die meisten Verfasser halten die Aspergillose für eine Mykose endogener Herkunft, da ihr Krankheitserreger auch im Mund gesunder

* Hier sprechen wir Frau Dr. A. CSILLAG für ihre Unterstützung bei der Identifizierung der Pilze unseren Dank aus.

Menschen oft nachweisbar ist. Demnach muss die Erkrankung in den vorliegenden Fällen — die endogene Herkunft auch bei Ratten annehmend — für eine unter dem Einfluss des Cortisons auftretende konsekutive Mykose erachtet werden.

Obwohl wir aus den Versuchen keine weitgehenden Schlussfolgerungen ziehen wollen, halten wir es auf Grund der Befunde notwendig, die Aufmerksamkeit auf die Rolle des Cortisons beim Zustandebringen von Pilzinfektionen

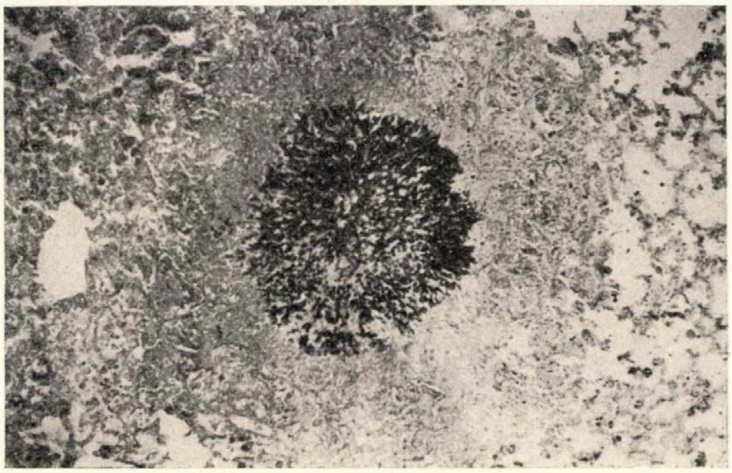

Abb. 1. Pilzkolonie im Zentrum des Lungenabszesses (MACMANUS—HOTCHKISS-Färbung 170×)

zu lenken. Es muss erwähnt werden, dass neuerdings auch Kliniker (ANTOPOL [1]) über infolge Cortisonbehandlung entstandenen systematischen Mykosen berichten. Wahrscheinlich wird durch gleichzeitige Verabreichung von Antibiotikum und Cortison die Möglichkeit der Pilzinfektion noch mehr gesteigert.

Zusemmenfassung

Infolge Cortisonbehandlung entstanden mykotische und bakterielle Lungenabszesse bei Ratten. Auf Wirkung von 5-mg-Dosen gingen 56,5% der Tiere an Lungenaspergillose zugrunde. Die Verfasser machen die hohen Cortisondosen für die Entstehung der Infektion verantwortlich.

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АСПЕРГИЛЛЕЗ ЛЕГКИХ, РАЗВИВАЮЩИЙСЯ У КРЫС ПОД ДЕЙСТВИЕМ ОБРАБОТКИ КОРТИЗОНОМ

Т. ШАГИ и К. ЛАПИШ

Резюме

Вследствие обработки кортизоном у крыс развивались грибковые и бактериальные абсцессы легких. Под действием доз в 5 мг 56,5% животных погибало от аспергиллеза легких. Причиной развития инфекций авторы считают большие дозы кортизона.

ÜBER DEN STICKSTOFF- UND KOHLENHYDRAT-STOFFWECHSEL DER FERMENTORKULTUREN VON ACTINOMYCES GLOBISPORUS STREPTOMYCINI (STREPTOMYCES GRISEUS)

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Eine Veränderung der Bestandteile des streptomycinerzeugenden Mikroorganismennährbodens in Tieffermentorkulturen ist in der Literatur nicht bekannt. Bisher wurden nur die Resultate von Schüttelkolbenversuchen beschrieben [1, 2]. Im Hinblick darauf, dass sich die Züchtung im Schüttelkolben mit seinem kleinen Volumen und der unzureichenden Ventilation von der Züchtung in den mehrere Liter fassenden Laboratoriums- oder viele Kubikmeter enthaltenden Betriebsfermentoren wesentlich unterscheidet und der Stoffwechsel der Mikroorganismen stark von den äusseren Verhältnissen abhängig ist, wollen wir unsere im Institut und in seiner Versuchsfabrik im Laufe anderthalbjähriger Arbeit gewonnenen Erfahrungen im Zusammenhang mit der Züchtung in Laboratoriums-Glasfermentoren zu 10—25 Liter und in 10 m^3 fassenden Betriebs-Eisenfermentoren nachfolgend mitteilen.

Bei diesen Versuchen sollten 3 Fragen geklärt werden: 1. Lässt sich in den guten Fermentorkulturen in den Veränderungen des Ammoniak-, gelösten organischen Stickstoff- und Kohlenhydratgehalts des Nährbodens im Laufe der Fermentation eine charakteristische Gesetzmässigkeit beobachten?

2. Falls derartige Gesetzmässigkeiten vorhanden sind, können wir dann aus ihrer Kenntnis irgendwelche Aufklärungen über die Bedingungen der zweckmässigen Streptomycinerzeugung gewinnen und sind 3. von den normalen Fermantationen abweichende charakteristische Stoffwechselstörungen der streptomycinerzeugenden Mikroorganismen zu beobachten?*

Substanzen und Methoden

Die im Institut verwendeten Stämme waren die von LOVREKOVICH durch Bestrahlung zu höherer Produktion gebrachten Abkömmlinge eines Actinomyces globisporus streptomycini Stammes. Als Nährboden diente ein Gemisch von 2% Sojamehl, 2% Glukose, 0,4% Maisextrakt (corn steep liquor) und 0,1% $CaCO_3$ [3]. In den Laboratoriumsfermentoren war das Anfangs-pH 7,0; in den Betriebsfermentoren wurde statt Glukose Stärke verwendet,

* DR. LOVREKOVICH, DR. HORVÁTH und DR. OBERRECHT, die das Fermentationsmaterial freundlicherweise zur Verfügung stellten, möchte ich auch an dieser Stelle meinen Dank aussprechen.

$CaCO_3$ bei mehreren Fermentationen weggelassen und auch das Anfangs-pH nicht auf 7,0 eingestellt, so dass die Fermentation meist bei einem Wert von 6,0 begann. In der durch Zentrifugieren vom Myzelium befreiten Fermentationsflüssigkeit wurde Ammoniak nach VAN SLYKE und CULLEN [4], Stickstoff nach der KJELDAHL-Methode im WAGNER-PARNASSschen Apparat und Zucker nach Eiweissfällung nach BERTRAND bestimmt. Im Betriebsfermentor, in dem wir als Kohlenhydratquelle statt Glukose Stärke verwendeten, wurden die Fermentationsflüssigkeiten vor der Zuckerbestimmung einer 10%igen salzsauren Hydrolyse unterworfen. Das pH wurde mit Glaselektroden gemessen, die auf den Abbildungen mitgeteilten Streptomycinwerte entsprechen den bei der biologischen Bestimmung gewonnenen Resultaten.

Experimenteller Teil

Die in Laboratoriums-Glasfermentoren in guten Fermentationen beobachteten typischen Veränderungen des Nährbodens sind Abb. 1 zu entnehmen.

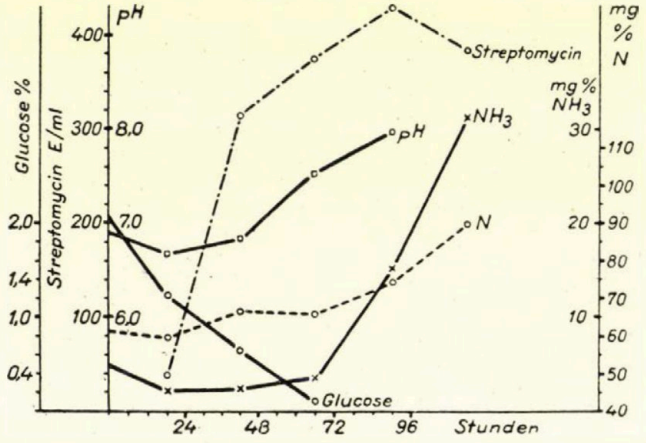

Abb. 1. Veränderung der Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwerte im Nährboden. Mittelwert von 13 Glasfermentationen. Intensiver Zuckerverbrauch, intensive pH- und NH_3-Erhöhung am Ende der Fermentation. Geringe Veränderung des org. N-Wertes in den ersten Tagen. Hoher Streptomycinwert

Abb. 1 zeigt die Durchschnittwerte von 13 Fermentationen. Bezeichnend für die guten Glasfermentationen ist der starke Glukoseverbrauch. Am 3. Tage wurde der Nährboden in diesen Fermentationen praktisch glukosefrei. Die Ammoniakwerte sanken anfangs ; nachdem jedoch die Glukose aus dem Nährboden verbraucht war, stieg der Ammoniakwert am 4. Tage plötzlich stark an. Dementsprechend war das pH des Nährbodens am Ende der Fermentation im Mittelwert nahe 8,0. In den organischen Stickstoffwerten trat in den ersten Tagen kaum eine Veränderung ein, erst als die Autolyse des Myzeliums begann, stieg dieser Wert. Die geringe Veränderung der Mittelwerte des organischen Stickstoffs während der ersten Tage der Fermentation, wo die grosse Myzelienmasse aufgebaut wird, deutet darauf hin, dass der Mikroorganismus anlässlich

der Fermentorzüchtung eine wirkungskräftige exozelluläre Protease erzeugt, wodurch aus dem unlöslichen Sojamehl kontinuierlich niedermolekulärer gelöster Stickstoff befreit wird, der den vom Myzelium eingebauten gelösten Stickstoff substituiert [5]. Die im Durchschnittswert 421 μg/ml Streptomycin ergebende

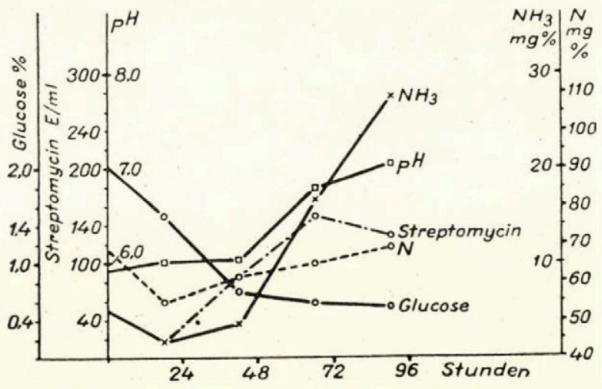

Abb. 2. Veränderung der Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwerte im Nährboden. 2% Stärke. Mittelwert von 10 Eisenfermentationen. NH_3 nahm bereits am dritten Tage plötzlich zu. Der Glukosewert nahm vom 2. Tage an kaum ab. Der org. N-Wert wurde in den ersten Tagen etwas niedriger. Geringer Streptomycinwert

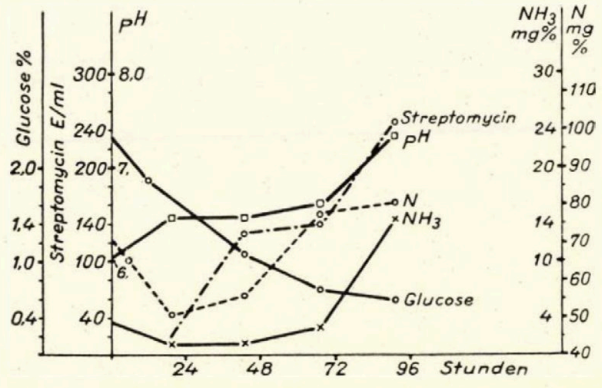

Abb. 3. Veränderung des Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwertes im Nährboden. 2,5% Stärke. Mittelwert von 10 Eisenfermentationen. Die plötzliche Erhöhung des NH_3-Wertes trat erst am 4. Tage ein. Der Glukosewert ist erst vom 3. Tage an wenig veränderlich. Der org. N-Wert nimmt an den ersten beiden Tagen stark ab. Erheblich höherer Streptomycinwert

Produktion war bei den damals im Institut benutzten Stämmen als sehr günstige Ausbeute zu betrachten.

Abb. 2 und 3 zeigen die Veränderungen der Nährbodenbestandteile in Eisenfermentoren von 10 m³ im Laufe der Fermentationen.

Abb. 2 und 3 geben die Mittelwerte von je 10 Fermentationen wieder. Bei den auf Abb. 2 angeführten Fermentationen enthielt der Nährboden 2%,

bei den auf Abb. 3 angegebenen Fermentationen 2,5% Stärke. In beiden Fermentationsserien hörte der Kohlenhydratverbrauch bei dem Wert von etwa 0,5% auf. Diese Erscheinung hat folgende Ursache: Das durch Hydrolyse erschlossene Kohlenhydrat stammt teils aus der Stärke, teils aus dem Sojamehl. Das letztere vermag der Mikroorganismus nicht zu verwerten. Bei den Versuchen im Glasfermentor war der Umstand, dass Sojakohlenhydrat nicht verbraucht wurde, nicht sichtbar, da die Kohlenhydratbestimmung ohne Hydrolyse erfolgte. Mit Hydrolyse lässt sich aber auch hier etwa 0,5% nicht verbrauchtes Kohlenhydrat nachweisen. Bei den Fermentationen mit 2% Stärke bleibt der Wert

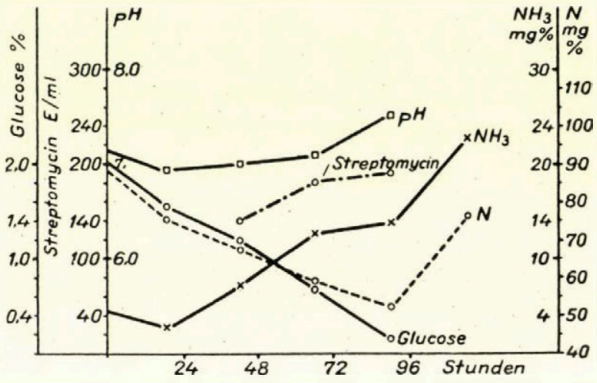

Abb. 4. Veränderung des Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwertes im Nährboden. Mittelwert von 10 Glasfermentationen. Schwach geröstetes Sojamehl. Ständig zunehmende Senkung des org. N-Wertes bis zum 4. Tage. Träger Glukoseverbrauch. Frühzeitige Erhöhung des NH_3-Wertes. Mittelmässiger Streptomycinwert

des verwendbaren Kohlenhydrats schon vom 2. Tage an fast unverändert, während bei den Fermentationen mit 2,5% Stärke eine geringe Abnahme des Kohlenhydratgehaltes erst vom dritten Tage an beobachtet werden kann. Die plötzliche Erhöhung der Ammoniakwerte trat bei den Fermentationen mit 2% Stärke 1 Tag früher ein als im Närhboden mit 2,5% Stärke. Der mehr Kohlenhydrat enthaltende Nährboden war für die Streptomycinerzeugung günstiger. Die Produktion stieg im Mittelwert um etwa 50%. Die Werte des organischen Stickstoffs verminderten sich an den ersten beiden Tagen der Fermentation in dem mehr Kohlenhydrat enthaltenden Nährboden kräftiger als in dem weniger Kohlenhydrat aufweisenden.

Nachfolgend teilen wir die Nährbodenveränderungen bei 2 Fermentationsstörungen mit. Die erste Störung entstand, als der Betrieb ein schwach geröstetes Sojamehl verwendete. Im Eisenfermentor des Betriebs wurde auf diesem Nährboden überhaupt kein Streptomycin erzeugt. Der Ammoniakwert stieg schon am ersten Tage der Fermentation auf 16 mg%. Als wir dieses Sojamehl in Laboratoriums-Glasfermentoren benutzten, kam es zu einer — wenn auch ver-

minderten — Streptomycinerzeugung. Um das Wesen dieser Fermentationsstörung kennenzulernen, nahmen wir im Glasfermentor 10 Fermentationen mit diesem ungenügend gerösteten Sojamehl sowie 13 Fermentationen in der Weise vor, dass wir dieses Sojamehl vorher nochmals rösteten. Die Durchschnittswerte der Nährbodenveränderungen sind Abb. 4 und 5 zu entnehmen.

Es wurde folgendes beobachtet: Während bei den guten Fermentationen der organische Stickstoff sich in den ersten Tagen entweder wenig veränderte oder bei starker Verminderung die Abnahme am 3. Tage bereits aufhörte und statt dessen Erhöhung eintrat, verminderte sich der Stickstoffgehalt des Nähr-

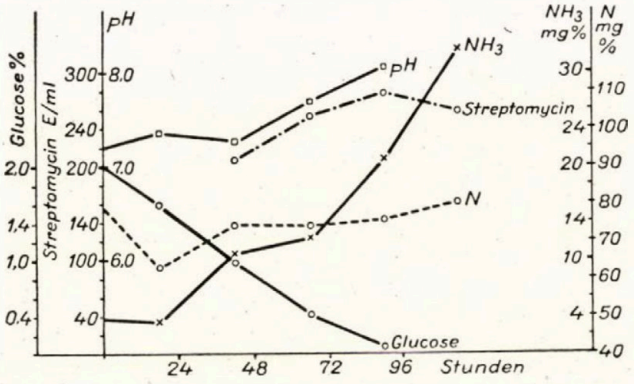

Abb. 5. Veränderung des Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwertes im Nährboden. Mittelwert von 13 Glasfermentationen. Stärker geröstetes Sojamehl. Die ständige Senkung des org. N-Wertes hat aufgehört. Intensiverer Kohlenhydratverbrauch. Höherer Streptomycinwert

bodens bei diesen Fermentationen immer mehr auch noch am dritten, ja selbst noch am vierten Tage. Der Mittelwert der Verminderung betrug am vierten Tage im Vergleich zum Ausgangswert 41%. Als wir die Differenz zwischen den Werten der 0. Stunde des 4. Tages mathematisch-statistisch untersuchten, fanden wir, dass der Stickstoff-Mittelwert in der 0. Stunde 87,9 mg betrug, mit einem Standardfehler von $\pm$ 6,16 mg%. Am 4. Tage war der Mittelwert 51,1 $\pm$ 4,94 mg%. Die Differenz der beiden Mittelwerte ist signifikant ($P < 0{,}01$). Als wir jedoch diese Fermentationen nach erneutem Rösten des Sojamehls in weiteren 13 Fällen durchführten, hörte diese allmähliche Verminderung des Stickstoffgehaltes, wie aus Abb. 5 deutlich hervorgeht, im Laufe der Fermentation im allgemeinen auf.

Diese Versuche ergaben demnach, dass bei Verwendung von schwach geröstetem Sojamehl im Nährboden der Gehalt an organischem Stickstoff in erheblich grösserem Ausmass und dauerhafter abnimmt als im Nährboden mit stärker geröstetem Sojamehl. Diese Erscheinung könnte darauf zurückgeführt werden, dass der Mikroorganismus in dem schwach geröstetes Sojamehl ent-

haltenden Nährboden mehr stickstoffhaltige Substanzen verbrauchte als in dem Nährboden mit stärker geröstetem Sojamehl. Doch könnte auch angenommen werden, dass das schwach geröstete Sojamehl irgendeine Hemmungssubstanz enthält, welche die Aktivität des exozelluläres Eiweiss spaltenden Enzyms der Streptomyces verhindert, so dass dieses Enzym aus dem unlöslichen Sojaeiweiss keinen gelösten Stickstoff abzuspalten vermag, der den ständigen Ersatz der von den Actinomyces verbrauchten organischen gelösten stickstoffhaltigen Substanzen darstellen würde. Die hochgradige Verminderung des Stickstoffgehaltes bei Verwendung des schwach gerösteten Sojamehls wäre demnach darauf zurückzuführen, dass der Mikroorganismus nicht mehr stickstoffhaltige Substanzen verbraucht, sondern, da die Aktivität des exozellulären eiweissabbauenden Enzyms gehemmt is, auf die im Nährboden ursprünglich anwesenden gelösten stickstoffhaltigen Substanzen angewiesen ist, wodurch deren erhebliche Verminderung zustande kommt.

Der letztere Fall kann aber nicht zutreffen, da die Wirkung des exozellulären eiweissabbauenden Enzyms bei Verwendung beider Sojamehlarten in gleicher Weise anzutreffen war.

Nachfolgend beschreiben wir zwei derartige Versuche: 1. Zu je 30 ml schwächer bzw. stärker geröstetes Sojamehl enthaltendem sterilisiertem Fermentationsnährboden gaben wir bei pH 7,0 je 50 mg exozelluläres eiweissspaltendes Enzym, hergestellt aus Actinomyces globisporus streptomycini-Fermentationsflüssigkeit [5]. Zur Bestimmung des Reststickstoffs wurden Proben entnommen und die Kolben bei 37° C für 24 Stunden in das Thermostat gestellt. Hiernach entnahmen wir erneut Proben zur Bestimmung des Reststickstoffs. Die Proben wurden zentrifugiert ; zu 5 ml Zentrifugat gaben wir 1,6 ml 20%ige Trichloressigsäure. Nach der Filtration wurde der Stickstoff bestimmt. Bei Anwendung von stark geröstetem Sojamehl war der Reststickstoffwert in der 0. Stunde 45,3 mg%, in der 24. Stunde 103,0 mg%, die Zunahme demnach 57,7 mg%. Bei Verwendung des wenig gerösteten Sojamehls betrug der Reststickstoffwert in der 0. Stunde 43,4 mg%, in der 24. Stunde 92,1 mg%, die Erhöhung 48,7 mg%.

2. Der Versuch wurde ähnlich durchgeführt wie der erste ; der Unterschied bestand lediglich darin, dass der Nähboden nicht sterilisiert wurde. Bei Benutzung des stark gerösteten Sojamehls betrug der Reststickstoffwert in der 0. Stunde 49,2 mg%, in der 24. Stunde 85,6 mg%, die Zunahme also 36,4 mg%. Bei Verwendung des schwach gerösteten Sojamehls war der Reststickstoffwert in der 0. Stunde 44,8 mg%, in der 24. Stunde 82,3 mg%, so dass die Zunahme 37,5 mg% ausmachte.

Ein grösserer Unterschied in bezug auf die Wirkung der exozellulären Protease trat bei den zwei verschiedene Sojamehlarten enthaltenden Nährböden in diesen Versuchen nicht in Erscheinung, so dass die Annahme, das schwach geröstete Sojamehl enthalte eine die Wirkung der Protease dieser Actinomyces

hemmende Substanz, experimentell nicht bestätigt werden konnte. Demnach muss die Erscheinung, dass bei fortschreitender Fermentation der Stickstoffgehalt in dem schwach geröstetes Sojamehl enthaltenden Nährboden ständig abnimmt, mit grösster Wahrscheinlichkeit damit erklärt werden, dass die Mikroorganismen die stickstoffhaltigen Substanzen in diesem Falle in erhöhtem Masse verbrauchen. Bei Verwendung des stärker gerösteten Sojamehls vermindert sich dagegen der Verbrauch der stickstoffhaltigen Substanzen.

Mit dieser Erklärung stimmt gut die Erscheinung überein, dass der Kohlenhydratgehalt in dem schwach geröstetes Sojamehl enthaltenden Nährboden

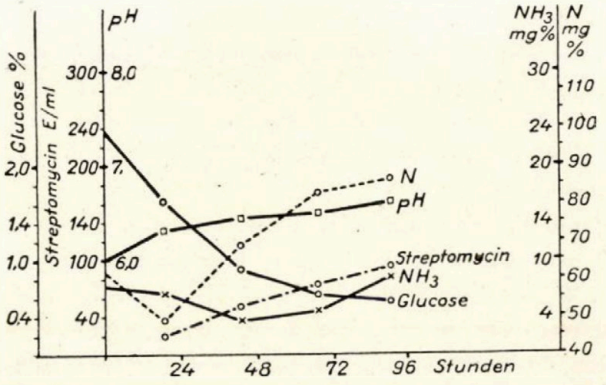

Abb. 6. Veränderung des Glukose-, NH_3-, gelösten org. N-, pH- und Streptomycinwertes im Nährboden. Mittelwert von 10 Eisenfermentationen. Rasche Abnahme des Glukosewertes in den ersten Tagen. Der org. N-Wert nimmt schon am 2. und 3. Tage stark zu. Niedrige pH- und NH_3-Werte. Geringe Streptomycinerzeugung

mit fortschreitender Fermentation im Mittelwert weniger abnahm als bei Verwendung des stärker gerösteten Sojamehls. In dem Nährboden mit schwach geröstetem Sojamehl wies der Glukoseverbrauch folgende Durchschnittswerte auf: Nach einem Tage 1,55%, nach zwei Tagen 1,18%, nach drei Tagen 0,67%, nach vier Tagen 0,19%. Bei dem stärker geröstetes Sojamehl enthaltenden Nährboden betrugen die Mittelwerte: nach einem Tage 1,57%, nach zwei Tagen 0,95%, nach drei Tagen 0,36%, nach vier Tagen 0,03%.

Die Signifikanz der Differenz in der Intensität des Zuckerverbrauches zwischen den beiden Fermentationen untersuchten wir folgendermassen. Wir errechneten in beiden Fällen die Differenz zwischen dem Zuckerwert am ersten und zweiten Tage der Fermentation. Bei Anwendung des schwach gerösteten Sojamehls betrug dieser Wert 0,37% $\pm$ 0,059%. Bei dem stärker geröstetes Sojamehl enthaltenden Nährboden ergab die Differenz 0,63% $\pm$ 0,095%. Die Differenz der beiden Mittelwerte war bei $P < 0{,}05$ signifikant.

Die Streptomycinerzeugung nahm bei Verwendung des stark gerösteten Sojamehls, nachdem sich der Stickstoff- und Kohlenhydratverbrauch normali-

siert hatte, im Mittelwert um 47% zu. Bei den mit schwach geröstetem Sojamehl durchgeführten Fermentationen beobachteten wir also eine Stoffwechselveränderung des Actinomyces globisporus streptomycini, für welche erhöhter Stickstoff- und verminderter Kohlenhydratverbrauch charakteristisch war.

Es sei noch erwähnt, dass bei dieser Fermentationsstörung auch die Veränderung des Ammoniakwertes in mehreren Fällen vom Üblichen insofern abwich, als dieser auch vor dem Verschwinden der Glukose aus dem Nährboden stieg. Dies deutet wahrscheinlich auf gesteigerte Desaminierung. Diese Erscheinung hörte auch nach stärkerem Rösten des Sojamehls nicht auf, so dass die auf der Abbildung angegebenen Mittelwerte schon in der 48. Stunde erheblich höher waren als der Ausgangswert.

Für die zweite Fermentationsstörung im Betrieb war neben der sehr niedrigen Streptomycinerzeugung charakteristisch, dass die Fermentationsflüssigkeit bei der Verarbeitung nur sehr schwer filtriert werden konnte und trübe blieb. Den Mittelwert der Veränderungen von 10 derartigen Fermentationsnährböden zeigt Abb. 6.

Während sich bei den auf Abb. 3 angeführten Fermentationen im gut erzeugenden 10 m^3-Fermentor die Stickstoffverminderung des Nährbodens im allgemeinen auf 2 Tage nach der Umimpfung erstreckte und im Mittelwert 30 bzw. 24% betrug, war eine Senkung des gelösten Stickstoffgehaltes im Nährboden bei diesen Fermentationen nur am ersten Tage wahrnehmbar. Auch diese betrug im Mittelwert nur 21%. Im Mittelwert des zweiten Tages trat bereits keine Senkung, sondern eine 10,5%ige Erhöhung ein ; am dritten Tage stieg diese Erhöhung auf 33,8%. Bei guten Fermentationen betrug die Zuhanme an organischem Stickstoff am dritten Tage im Mittelwert nur 7%.

Die Veränderung des Ammoniakwertes erfolgte nicht einheitlich. Bei 3 von 7 Fermentationen trat die übliche mehr als 10 mg%ige Erhöhung am Ende der Fermentation nicht ein. Der Durchschnittswert der Ammoniakwerte war am vierten Tage 8,06 mg%, während bei den auf Abb. 3 angegebenen Betriebsfermentationen der Mittelwert am vierten Tage 14,63 mg% betrug. Bezeichnend für diese Fermentationen war ferner, dass der anfängliche pH-Wert von ungefähr nur bei 2 von 10 Fermentationen am Ende der Fermentation über den gewohnten pH-Wert 7 stieg. In 8 Fällen wurde dieser Wert nicht erreicht, so dass bei Beendigung der Fermentation der pH-Mittelwert aller Fermentationen 6,59 ausmachte. Der Zuckerverbrauch betrug am zweiten Tage nach dem Beginn im Mittelwert 0,89%, während der Mittelwert des gleichen Tages bei den auf Abb. 3 angeführten guten Fermentationen 1,16% ergab.

Da die Mikroorganismen an den ersten 3 Tagen morphologisch keine Anzeichen eines Zerfalls aufwiesen, besteht kaum die Möglichkeit, an infolge Autolyse freigewordenen gelösten Stickstoff zu denken. Eine der Fermentationen untersuchten wir auf exozelluläre Protease und vermochten diese auch nachzuweisen.

Zur mathematisch-statistischen Untersuchung erwies sich nur das Ausmass des Kohlenhydratverbrauchs als ausreichend. Wir untersuchten die Differenz zwischen den Mittelwerten der Glukosewerte am zweiten Tage der Fermentation sowohl bei guten Fermentationen im Eisenbehälter als auch bei den vorliegende Stoffwechselstörung aufweisenden Fermentationen. Während der Glukose-Mittelwert, wie wir schon erwähnten, am zweiten Tage 1,16% $\pm$ $\pm$ 0,17% ausmachte, erhielten wir bei diesen Stoffwechselstörung aufweisenden Fermentationen einen Mittelwert von 0,89 $\pm$ 0,17%. Unter Berücksichtigung aller Kautelen fand die medizinische Abteilung des Instituts für angewandte Mathematik (JUVANCZ) diese Differenz signifikant, so dass es berechtigt erscheint, einen im Vergleich zum Normalen erhöhten Kohlenhydratverbrauch anzunehmen.

Der bei dieser Fermentationsstörung beobachtete Kohlenhydratverbrauch und die Veränderung im Stickstoffwert stellen gerade das Gegenteil von dem dar, was wir bei der durch Anwendung von schwach geröstetem Sojamehl verursachten Fermentationsstörung beobachtet hatten. Im letzten Fall war die Verminderung des organischen Stickstoff-Wertes gesteigert und erstreckte sich über mehrere Tage ; der Zuckerverbrauch war gering. Im ersten Fall war der Zuckerverbrauch gesteigert, und es dominierte die Erhöhung des organischen Stickstoffniveaus.

Zur Erklärung dieser Fermentationsstörung denken wir neben erhöhtem Zuckerverbrauch an eine verminderte Desaminierung. Darauf dürfte die Vermehrung der stickstoffhaltigen gelösten Substanzen im Nährboden, der niedrige pH-Wert und das Ausbleiben der Erhöhung des Ammoniakwertes zurückzuführen sein.

Der Leiter der Versuchsfabrik vermochte die Fermentatiosstörung dadurch zu beheben, dass er dem Nährboden erneut Kalziumkarbonat zufügte, das auf Grund von Literaturangaben bei der üblichen Zusammenstellung des Nährbodens weggelassen worden war.

Da das anorganische Phosphat neben Verminderung der Streptomycinerzeugung erhöhten Zuckerverbrauch verursacht [6] und Kalziumkarbonat das Niveau an löslichem Phosphat herabsetzt [2], ist es möglich, dass diese Stoffwechselstörung von dem für den Mikroorganismus hohen Phosphatniveau hervorgerufen wurde, das durch Zugabe von Kalziumkarbonat auf einen günstigeren Wert sank.

Besprechung

Aus den Untersuchungen ging hervor, dass bei der Züchtung des Actinomyces globisporus streptomycini im Fermentor das im Sojamehl befindliche Kohlenhydrat vom Mikroorganismus nicht verbraucht wird. Nach Abschluss unserer diesbezüglichen Untersuchungen erschien die Mitteilung von HOCKENHULL und

Mitarbeitern [7], die bei der Züchtung einer Albus-Varietät des Mikroorganismus mit sehr hoher Streptomycinerzeugung im Schüttelkolben ebenfalls festgestellt hatten, dass die Kohlenhydrate des Sojamehls, Stachyose, Raffinose und Sukrose, von ihrem Stamm nicht verbraucht wurden.

Aus den mitgeteilten Untersuchungsergebnissen geht hervor, dass die ausreichende Menge des verwendbaren Kohlenhydrats und die entsprechende Intensität des Kohlenhydratverbrauches bei diesen Actinomyces-Stämmen unseres Instituts eine sehr wichtige Voraussetzung der guten Streptomycinerzeugung darstellen. Dies wird auch dadurch bewiesen, dass die Streptomycinerzeugung um etwa 50% zunahm, als wir anfangs die Kohlenhydratkonzentration im Nährboden des Eisenfermentors von 2% auf 2,5% erhöhten. Als wir jedoch als Nährboden schwach geröstetes Sojamehl benutzten, nahm die Intensität des Zuckerverbrauches ab, während der Verbrauch der stickstoffhaltigen Substanzen stark zunahm. Die Streptomycinerzeugung war mittelmässig. Als wir das Sojamehl stärker rösteten, sank der Stickstoffverbrauch, stieg der Kohlenhydratverbrauch und nahm die Streptomycinerzeugung zu. Drittens war im allgemeinen bei der Erreichung eines hohen Erzeugungsniveaus der Kohlenhydratverbrauch stets intensiv.

Wenn hingegen dem intensiven Kohlenhydratverbrauch kein paralleler Verbrauch der stickstoffhaltigen Substanzen folgte, wie bei der zweiten Fermentationsstörung gefolgert werden konnte, so dass sich deren Menge im Nährboden ständig vermehrte, war die Ausbeute an Streptomycin gering. Als Gesamtergebnis dieser Untersuchungen darf angenommen werden, dass eine gute Streptomycinerzeugung mit intensivem Kohlenhydratstoffwechsel einhergeht, aber parallel damit auch eine kräftige Desaminierung erfolgen muss.

Nachdem diese Untersuchungen mit Stämmen vorgenommen wurden, die im Betriebsfermentor auf stärkehaltigem Nährboden nicht mehr als 300 E/ml produzierten, während im Laboratoriums-Glasfermentor auf glukosehaltigem Nährboden häufig auch höhere Werte als 500 E/ml vorkamen, ergab sich die Frage, ob nicht auch bei besser erzeugenden Stämmen den hier beschriebenen ähnliche Nährbodenveränderungen entstehen. Wir hatten Gelegenheit, in einem der Betriebe die Nährbodenveränderungen von Stämmen, die eine erheblich höhere Streptomycinerzeugung aufwiesen, als die von uns verwendeten, ebenfalls auf sojamehlhaltigem Nährboden zu beobachten. Die Veränderung der Mengen von Stickstoff, Ammoniak und Kohlenhydrat fand in ähnlicher Weise statt, wie es oben beschrieben wurde. Das Kohlenhydrat im Sojamehl wurde von diesem Stamm als Nährmittel ebenfalls nicht verbraucht. In der Fermentationsflüssigkeit konnte gleichfalls eine stark wirkende exozelluläre Protease nachgewiesen werden.

Zusammenfassung

Die Veränderungen der Ammoniak-, organischen Stickstoff-, Kohlenhydrat-, pH- und Streptomycinwerte bei Anwendung von Sojamehl, Maisextrakt, Glukose bzw. Stärke enthaltendem Nährboden anlässlich der Züchtung in Laboratoriums-Glas- und Betriebs-Eisenfermentoren mit guter und schwacher Streptomycinerzeugung werden besprochen.

Für gute Fermentationen ist ein intensiver Kohlenhydratverbrauch charakteristisch ; die plötzliche Erhöhung der Ammoniakwerte tritt erst nach Verbrauch des verwendbaren Kohlenhydrats ein. Der organische Stickstoffwert nimmt in den ersten Tagen der Fermentation vielfach nur wenig ab, im Stadium der Autolyse dagegen zu. Der pH-Wert steigt allmählich an. Die Kohlenhydrate des Sojamehls werden vom Mikroorganismus als Nährstoff nicht verbraucht.

Auf dem schwach geröstetes Sojamehl enthaltenden Nährboden waren verminderter Kohlenhydratverbrauch und die gesteigerte, lange Zeit dauernde Verminderung des gelösten Stickstoffs — bei mässiger Streptomycinerzeugung — kennzeichnend. Wurde das Sojamehl stärker geröstet, hörte der gesteigerte Stickstoffverbrauch auf, der Kohlenhydratverbrauch stieg, die Streptomycinerzeugung nahm zu.

Für eine andere Betriebsfermentationsstörung war charakteristich, dass Kohlenhydrat stärker als normal verbraucht wurde und daneben auch der Gehalt an organischem Stickstoff sehr rasch zunahm. Die starke Erhöhung des Ammoniakwertes blieb mehrmals aus, die Alkalisierung des Nährbodens war erheblich geringer als bei ungestörter Fermentation.

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ДАННЫЕ АЗОТИСТОГО И УГЛЕВОДНОГО ОБМЕНА КУЛЬТУР ACTINOMYCES GLOBISPORUS STREPTOMYCINI (STREPTOMYCES GRISEUS) В ФЕРМЕНТАТОРАХ

Ш. ШИМОН

Р е з ю м е

Автор описывает изменения содержания аммиака, органического азота, углевода, pH и стрептомицина в питательной среде при хорошем и слабом производстве стрептомицина, в случае лабораторного и заводского культивирования в стекляных и железных ферментаторах, применяя питательную среду из соевой муки, кукурузной гущи, глюкозы или крахмала.

Хорошая ферментация характеризуется интенсивным потреблением углевода, внезапное повышение содержания аммиака наступает только после израсходования используемого углевода. Содержание органического азота в течение первых дней ферментации часто только немного понижается, а в стадии автолиза — повышается. pH постепенно повышается. Углеводы соевой муки микроорганизм не использует в качестве питательного вещества.

Среда из слегка жженой соевой муки характеризовалась пониженным потреблением углевода и длительным убыванием растворенного азота, при умеренном производстве стрептомицина. В случае применения более поджаренной соевой муки повышенное потребление азота прекратилось, повысилось потребление углевода и повысилось производство стрептомицина.

Другая производственная ферментационная аномалия характеризовалась сверхнормальным потреблением углевода, причем быстро увеличивалось содержание органического азота. Часто отсутствовало сильное повышение содержания аммиака, ощелачивание питательной среды было значительно слабее, чем при беспомешной ферментации.

STUDIES ON THE IMMUNOLOGICAL PROPERTIES OF SALMONELLA TYPHI STRAINS

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Although vaccines against typhoid fever have been in use since 1896, on grounds of the work by KOLLE and PFEIFFER [1], opinions still differ in several points. It has not been decided what properties make a strain particularly suitable for vaccine production and which vaccine may be expected to be more effective, the one prepared from bacterial antigen or the one prepared from extract antigen.

Opinions also differ as to the methods for estimating the potency of vaccines, though standardisation is obviously desirable.

LEISHMAN [2], in 1912, was the first to emphasize that highly virulent strains should be used for producing vaccines. Similar views have been expressed by PERRY et al. [3], ARKWRIGHT [4], GRINNEL [5], SILER et al. [6], FELIX et al. [7].

In general, the strains of *S. typhi* used for producing vaccine contain both the O and Vi antigens and are highly virulent for mice.

Recently, BATSON et al. [8] isolated an avirulent variant (42A58V) of the Panama carrier strain (42A58), used for vaccine production in the USA. This variant was morphologically, serologically, as well as in its simple biochemical properties identical with the virulent strain and proved to have a mouse protecting effect equivalent with that of the virulent strain.

After this discovery the possibility arose of obtaining by controlled mutation such avirulent, but highly immunogenic strains which would cause milder postinoculation reactions and could be used for vaccine production.

To elucidate this point, we have examined the immunogenic action of various strains of S. typhi and of extracts prepared from them. We have also investigated the properties of the variants obtained by exposing these strains *in vitro* to chloramphenicol until resistance to the antibiotic had developed.

We have also determined in what measure the extracts reflect the properties of the strains they have been prepared from. The latter problem is important in view of the fact that the nature of the antigen to be used for producing vaccine is another controversial issue in the literature. In Hungary and Poland [12] relying on the work of LOVREKOVICH and RAUSS [9] and RAUSS [10, 11],

the trichloracetic acid extract of BOIVIN is employed, while in the Soviet Union [13] the vaccine contains the antigen prepared according to RAISTRICK and TOPLEY [14], by digestion with trypsin and subsequent precipitation with acetone. In Switzerland [16] the GRASSET antigen obtained by freezing and thawing is used. In several other countries, on the other hand, bacterial vaccines are in use, on the reasoning that the extracts do not contain the complete immunogenic property of the bacterial cell. In view of this divergence in opinion we have examined the protective action of different strains and their extracts.

According to FELIX [17], the strains used for vaccine production should be virulent and should contain the O and the Vi antigens. FELIX, in disagreement with most authors (RAUSS *et al.* [18], BOIVIN [19, 20], LANDY [21], BATSON [22], HENDERSON [23, 24], MORGAN [23]), does not believe the mucin technique to be suitable for use in the assay of vaccines prepared from different strains. Therefore, it appeared important to examine the properties of different S. typhi strains with and without the use of mucin, employing a highly virulent S. typhi strain for challenging.

Materials and methods

S. typhi strains: Ty2; 42—A—58 (the Panama carrier strain); 42—A—58—V (the avirulent mutant of the former); Ty 300 (a laboratory strain) and the *in vitro* chloramphenicol-resistant variants of these strains (42—A—58 R, 42—A—58 VR, Ty 300 R). These were obtained by passaging the strains in broth containing increasing amounts of chloramphenicol.

Cultivation, preparation of antigen. The strains were grown under identical conditions in the DOLE [25] dialyzed medium, as modified by us [26]. The bacteria collected by centrifugation were killed with acetone and dried. The vaccines, as well as their trichloracetic acid extracts (by KABAT and MEYER's method [27]) were prepared from these standard bacterial powders. After having been extracted three times, the extracts were pooled, dialyzed at room temperature for 48 hours against running water and were dried at room temperature.

Methods of assay. In virulence tests, the strains were examined simultaneously by the modified method of RAUSS [18] (suspension in 5 per cent mucin), and by the method of FELIX [17], as modified by us. In the latter method 6 to 7 hour cultures on solid Hartley agar (instead of the 16 to 18-hour ones) were suspended in Ringer's solution and suspensions containing different quantities of germs were used for determining the intraperitoneal LD_{50} for mice. The LD_{50} of the strain Ty2 was found to vary between 10 to 20 germs by the mucin method, as compared to the 14,5 million germs by the FELIX method.

The bacterial and extract vaccines were assayed in decreasing volumes (0,02, 0,004 and 0,0008 ml for the bacterial vaccines and 20, 4 and 0,8 μg for the extracts). Twenty mice were inoculated subcutaneously with each dilution and 7 days later the animals were infected with equal doses of the mucinous, or of the mucin-free suspensions of strain Ty2. The infective Ty2 suspension was estimated to contain 1000 to 1500 LD_{50}.

The toxicity of extract antigen was determined by inoculating 10 mice intraperitoneally with each dilution, decreasing in geometrical progression from 5 mg/ml, seeking the dose causing 50 per cent mortality.

In order to work with equal germ counts, the suspensions containing 1000 million germs per ml, as determined by the MACFARLAND standard, were tested for nitrogen content according to KJELDAHL and suspensions of equal microbial counts have been used for immunisation.

The results were evaluated partly by the REED and MUENCH [28] formula for LD_{50}, partly by determining the potency ratio by means of probit-analysis.

Results

The 7 strains tested greatly differed in virulence for mice. As determined by both methods, equal microbial counts were required of strain Ty2 and strain 42A58 to kill the mice. Strain 42A58V was practically non-lethal for mice. The virulence of strains resistant to chloramphenicol *in vitro* was markedly diminished (VÁCZI, TOLNAI [36]).

Forty mice, weighing 12 to 13 g each, were inoculated with 0,5, 0,1, or 0,02 ml of vaccine, prepared from 7 strains of different virulence and containing equal amounts (0.020 mg/ml) of nitrogen. Seven days later, 20 mice in each group were infected with virulent Ty2 in mucin suspension, and 20 were given the suspension of virulent Ty2, prepared according to FELIX. The results for strains Ty2, 42A58 and 42A58V are presented in Table I.

Table I

Protective value of bacillary vaccines prepared from different strains

Designation of strain Method of infection	Potency ratio
Felix technique	
Ty2	1
42A58	$0{,}2 < 0{,}9 < 3{,}1$
42A58V	$0{,}1 < 0{,}6 < 2{,}3$
Mucin technique	
Ty2	1
42A58	$0{,}1 < 0{,}4 < 1{,}1$
42A58V	$0{,}1 < 0{,}2 < 0{,}4$

In agreement with the claim made by BATSON, it was found that the avirulent strain 42A58V had a definite mouse-protecting action, in both the mucin and the FELIX tests. Both methods showed the vaccine prepared from strain Ty2 to be the most potent. Apart from yielding results essentially identical with those obtained by the FELIX method, the mucin method had smaller limits of error in mouse protection tests (as determined by statistical analysis), and was more easily reproducible than were the tests without mucin.

Our results are in agreement with those obtained by BATSON et al. and confirm that no demonstrable relationship exists between the mouse virulence and immunogenicity of S. typhi strains.

The fact that both the 42A58 and the 42A58V strains contained O and Vi antigens and were in the S-phase, calls attention to the potential involvement in the determination of the lethal action of S. typhi on mice of factors

other than the occurrence of O and Vi antigens and the S form of bacteria. These factors are still unknown.

For unknown reasons, strains Ty 300 and Ty 300 R had no mouse protecting action.

Next the less virulent variants of strains 42A58 and 42A58V, rendered *in vitro* resistant to chloramphenicol (100μg/ml) were tested for immunogenicity, comparing them with the original, susceptible strains. Since in earlier tests the mucin method had proved suitable for such tests, the animals in this series were immunized by the mucin method. The results are shown in Table II. The high homogeneity of the results made it permissible to estimate LD_{50} simply by the REED—MUENCH method.

Table II

Protective value of different bacillary vaccines

Designation of strain	Volume in ml of dose affording 50 per cent protection
42A58	0,16
42A58R	0,10
42A58V	0,17
42A58VR	0,10

According to these data, the *in vitro* resistance to chloramphenicol had no effect on the mouse protecting potency of the strains. Thus, in strain 42A58R we obtained one which was less lethal for mice than, and had the same mouse protective power as, strain 42A58. This was encouraging evidence for the production of strains which might advantageously used for making bacillary vaccines.

Subsequently we studied the properties of bacteria, as well as of the extracts prepared from them. First of all, we determined the toxicity of BOIVIN extracts prepared by KABAT and MEYER's method from the 7 strains mentioned. In Table III the LD_{50} values are presented, as determined by the REED-MUENCH formula.

The data in Table III reveal that the above strains, which are highly different in both their protective and lethal effects on mice, yield extracts which kill mice at surprisingly similar rates. It is further interesting that the toxicity of the strains *in vitro* resistant to chloramphenicol was also similar.

In an experiment carried out in two steps it could be confirmed that extract antigens had an excellent mouse protective power. In one series of mouse protection tests extracts prepared from strains Ty2, Ty 300, 42A58

Table III

Toxicity of extracts from different strains

Designation of strain	LD_{50} in mg
Ty2	0,88
Ty300R	0,96
Ty300	1,03
42A58	0,77
42A58R	0,96
42A58V	0,96
42A58VR	1,15

and 42A58V were examined, while in the other series strains 42A58 and 42A58V, as well as the extracts prepared from the *in vitro* chloramphenicol-resistant variants of the latter strains. The results for part 1 are presented in Table IV.

Table IV

Protective value of extract vaccines prepared from different strains

Designation of strain	Potency ratio
Ty2	1
Ty300	$0{,}01 < 0{,}06 < 0\ 14$
42A58	$0{,}20 < 0{,}51 < 1{,}35$
42A58V	$0{,}20 < 0{,}79 < 3{,}38$

The calculated potency ratios show that the extracts prepared from strains 42A58 and 42A58 V afforded a protection equivalent with that producible with the Ty 2 extract, while the extract of strain Ty 300 was far less effective. This is the more remarkable, since according to the data in Table III, the extract of strain Ty 300 was inferior to none of the strains.

The results for part 2 of the experiment are shown in Table V.

Table V

Protective value of extract vaccines of different origin

Designation of strain	Vaccine affording 50 per cent protection Dose in mg
42A58	0,009
42A58R	0,012
42A58V	0,017
42A58VR	0,013

There was no difference in protective value between the strains *in vitro* resistant or susceptible to chloramphenicol.

Discussion

The strains of Salmonella typhi are called virulent or avirulent according to their being or not toxic for mice toxicity usually being associated with the S-R variation. According to MAALOE [29 a, b], virulence is composed of *infectivity*, *invasiveness* and *toxicity*. In mouse experiments only the two latter components of virulence are tested, since S. typhi is not a natural pathogen for mice. For this reason, it seems to be more justified to call the test a "mouse-killing test".

It is known that the simultaneous presence of O and Vi antigens and the S variant of bacteria do not necessarily indicate the presence of a lethal effect on mice. According to RAUSS, the concept of virulence cannot be explained on the basis of present knowledge and methods [30]. BATSON's [8] and our own experiments supplied evidence showing that the presence of the Vi antigen alone does not explain the virulence, or, to be more exact, the mouse-killing property, of S. typhi strains. Strains differing in virulence are not significantly different in immunogenic properties, though they may greatly differ in their Vi antigen content (VÁCZI and MIHÁLYFI [35]). Working with pure O and Vi strains, RAUSS [37] showed that the pure Vi strain is just as slightly virulent for mice, as the pure O strain. In virulent strains both antigens are present. Thus, virulence is a complex property, only partially directed by antigens. Both antigens follow the rules of serological specificity and, accordingly, only a homologous protection develops in animals immunized with pure O or pure Vi strains. Only the simultaneous inoculation of O and Vi antigens will induce massive protection against the strain with complete antigenic structure. The mouse LD_{50} cannot be relied upon in making our choice of S. typhi strains for producing vaccine.

In our experiments vaccines prepared from strains different in their O and Vi contents were tested and compared to one another in mouse protection tests with, respectively without mucin. The mouse protective effect of strain 42A58V was demonstrable in both tests, although this strain contains both O and Vi antigens, but does not kill mice.

Our investigation supplied evidence showing that there is no relationship between the toxicity of S. typhi extracts and mouse protective effect so that the extracts cannot be assayed on grounds of their *lethal action on mice*. Immunisation of mice with 20, 4, and 0,8 μg of dried extract and superinfection 7 days later are recommended for assaying extracts prepared by the method we have employed.

There are reports in the literature dealing with the common and different properties of Salmonella strains and their extracts. For example, MACKENZIE, PIKE and SWINNEY [31, 32] extracted from two S-type S. typhi murium strains of different virulence polysaccharide antigens not distinguishable by chemical methods. These authors are of the opinion that toxicity and immunogenicity are distinct properties of extracts, and are determined by different components of the cell. In agreement with our opinion, the above authors do not believe it either to be able to assay the antigenic value of extracts on the basis of their toxicity. In their study on S. typhi strains, CHERNOKHVOSTOV et. al. [33] found no relationship between the virulence of strains and the toxicity of the extracts prepared from them. BOIVIN [34], in his investigations into the properties of bacteria of the Salmonella group and of the extracts prepared from them, came to the conclusion that the extracted substances cannot be held responsible for the virulence of different strains. The changes produced in animals by bacteria differ from those produced by their extracts.

Further investigations may elucidate whether the virulence of S. typhi strains is bound to the functional property of the intact bacterial cell, or to some cellular component. We intend to determine in addition the kind of inoculation reaction a vaccine prepared from avirulent strains would produce.

Summary

The protective value of bacillary vaccines prepared from 7 strains of *S. typhi* has been examined. The strains differed in their lethal effect on mice. Four of them were susceptible to chloramphenicol and the other 3 were *in vitro* resistant to that antibiotic. The extracts prepared from the above strains have been tested for toxicity and the protective value of vaccines made of these extracts has been determined.

1. *S. typhi* strains of different toxicity for mice afforded considerable protection to mice against superinfection. The property providing protection is not related to the mouse toxicity of the strains. It is probable that factors other than the O and Vi antigens are also involved in the ability of the S-type *S. typhi* strains to kill mice.

2. Mouse protection tests made by the mucin technique yielded results comparable to those obtained by methods not involving the use of mucin. The mucin tests are readily reproducible and have smaller sources of error. Thus, the mucin method is suitable for use in vaccine assay.

3. The 7 strains, all of them highly different in their toxicity and protective effect on mice, supplied extracts which were almost identical in lethal action on mice.

4. Tests for toxicity do not seem suitable for assaying the protective potency of extracts. Immunisation of animals with extracts and subsequent protection tests seem to be more reliable for such assays.

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ИММУНОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ШТАММОВ SALMONELLA TYPHI

ДЬ. ТОЛЬНАИ, Л. ВАЦИ и ДЬ. БАРШИ

Резюме

Авторы исследовали защитную ценность бациллярного прививочного материала, изготовленного из 7 штаммов S. typhi, обладающих различной патогенностью для мышей. Из этого числа 4 штамма оказались чувствительными к хлорамфениколу, а 3 штамма были резистентны к нему in vitro. В дальнейшем исследовали токсичность экстрактов тех же штаммов, а также защитную ценность прививочных материалов, изготовленных из этих экстрактов.

1. Штаммы тифозных бактерий, обладающие различной патогенностью для мышей, в значительной степени защищали мышей от заражения. Свойство вырабатывающее иммунитет не связано с патогенностью штаммов для мышей. Вероятно, что патогенность для мышей штаммов S. typhi типа S, обладающих антигенами O и Vi, зависит не только от упомянутых антигенов, а также и от других факторов.

2. Проба на иммуногенность с применением муцина дает такой же результат, как исследования производимые без муцина, хорошо воспроизводится и имеет меньше источников ошибок. Следовательно способ с применением муцина пригоден для измерения ценности прививочных материалов.

3. 7 штаммов, различающихся между собой в большой мере по патогенными и иммуногенным свойствам для мышей, давали экстракты, обладающие почти одинаковой патогенностью для мышей.

4. Для измерения иммуногенного эффекта экстрактов исследование токсичности не оказывается подходящим. Вместо этого более надежным является иммунизация животных экстрактами и затем проведение пробы на иммуногенность.

THE PRODUCTION IN EXPERIMENTAL ANIMALS OF HIGH TITRE IMMUNE SERA USING INFLUENZA VACCINES MIXED WITH OIL ADJUVANTS

By

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Earlier investigations into the antigenic structure of influenza A strains [1, 2] provided evidence that finer distinctions among strains are best demonstrated in immune sera of high titre. The next task was to elaborate methods of immunisation permitting of the production of high titre immune sera in those small-sized laboratory animals (hamsters, rats) which, among other reasons, because of their poor immune response have not as a rule been used in research work concerned with the structure of antigens.

Data in the literature made it appear promising to admix an oil adjuvant to the virus and so to increase antibody production to antigens in the experimental animals. As early as 1916, lipoids were reported to have been used to increase the immune response [3]. The addition of adjuvants nonetheless remained uncommon until the studies of FREUND and MCDERMOTT [4]. Using horse serum, these authors found that on mixing it with killed tubercle bacilli suspended in paraffin oil, they obtained a higher precipitin titre in guinea pigs. With numerous bacteria [5, 6] and other antigens [7, 8] it proved likewise possible to attain high titres in animals by the addition of oil adjuvants. As regards viruses, those of rabies [9], poliomyelitis [10], and influenza, need to be mentioned. With the latter, FRIEDEWALD [11] was the first to apply the findings of FREUND and MCDERMOTT [4] with success to the immunisation of rabbits and ferrets. TSHALKINA [12] enhanced the immunogenic potential of the virus by adding *Mycobacterium butyricum*. The first human experiments with the inoculation of influenza virus vaccine mixed with an oil adjuvant were carried out by HENLE and HENLE [13]. Of recent years, SALK and co-workers succeeded in inoculating with good results first monkeys [14], then human subjects [15, 16, 17], in great numbers.

The experiments described below were undertaken to study the immunogenic effect of the purified influenza virus mixed with an oil adjuvant in different kinds of laboratory animals (hamsters, rabbits, ferrets and rats).

Materials and methods

Virus strains. Influenza A-prime England 1/51 (Liverpool, Lp) and Sweden 3/50.

Red cells. Chicken blood was obtained by heart puncture and immediately preserved in a modified Alsever's solution [18]. From the stored blood the erythrocytes were washed four times with physiological saline, immediately before using them.

Virus preparation. The strains were inoculated into the allantoic sac of chick embryos 11 days old. The fluid was drawn after incubation at 35° C for 48 hours. TAKÁTSY's method was used in purifying and concentrating the virus [19, 20].

Emulsification of the virus with oil. In this, the method of SALK [14] was followed. Nine parts of the mineral oil known as Bayol D were mixed with one part of the emulsifying agent called Arlacel A (mannide mono-oleate), allowed to stand for 20 minutes at 100° C, sterilized in boiling water, and brought together with an equal volume of the saline solution of the above virus preparation. Emulsification lasted for 20 to 25 minutes, and was effected by repeatedly withdrawing and ejecting the mixture with a 5-ml Record syringe through a 12-gauge cannula until a drop of it placed on the surface of water refused to spread thereon.

Method of inoculation ; blood drawing. On one, or with 2 to 3 weeks between, on two occasions hamsters and rats were inoculated with 0,25 ml, and rabbits and ferrets with 1 ml, of the oily emulsion ; the inoculum was deposited deep in the musculature of the thigh. Blood was drawn from the animals at various intervals in a mildly narcotic state. Controls were invariably inoculated with a virus suspension diluted in an equal volume of 0,9-per cent salt.

In the *haemagglutination (HA), haemagglutination-inhibition (HI), and complement-fixation (CF) tests,* TAKÁTSY's spiral loop micromethod on plexiglass plates was used [21].

One *HA unit* was considered to be equivalent to the contents of 1 ml of that virus dilution which yielded ++ haemagglutination in the test.

The *virus-neutralisation test* was carried out with the method of HORVÁTH [22]. To the 1 : 2 dilutions of the immune serum inactivated at 56° C for 30 minutes in the tubes, an equal volume of allantoic fluid containing 1000 LD_{50} doses of virus was added, and the mixture was incubated at + 4° C for 3 hours. Meanwhile, into every one of the phials in the revolving drum a quantity of 0,5 ml of nutrient fluid was measured, using an automatic pipette. To this were added, with the aid of a Pasteur pipette, 0,03 ml of a tissue suspension prepared from the minced chorioallantoic membrane of eggs incubated for 15 days. From each of the different samples of the serum-virus mixture 0,2 ml were measured into each of 4 phials, and the phials were then kept revolving for 60 hours at 35° C. The haemagglutinating capacity was thereafter titrated in the nutrient fluids. The serum 50-per cent neutralisation titre was calculated by the method of REED and MUENCH [23].

Experimental

Antibody response in hamsters after inoculation of influenza A-prime (Lp) virus in oil or of virus in saline. In the first experiment six hamsters each were inoculated with 3500 HA units of virus in oil adjuvant and saline, respectively. The development of the antibody response was kept under observation for 39 weeks in all. Thereafter, at various intervals blood samples were drawn and the titres estimated by HI, CF, and neutralisation tests. The arithmetic means of the titres of the two groups each comprising 6 animals are presented in Fig. 1.

Since the distribution curves for the individual animals run virtually the same course, they are all combined in this graph. In the group of hamsters that had received the virus in saline the antibody response was poor and of a short duration, whereas the antibody level for the hamsters inoculated with the adjuvant vaccine reached a much higher peak in the 5th week and maintained it even in the 18th week, with a slight drop showing as late as the 39th week.

The curves of titres for HI, CF, and virus-neutralising antibody run approximately the same course for every animal in the same group.

A second experiment was carried out to see whether, if given the adjuvant, 1/100th of the quantity of virus employed in the first experiment was likewise capable of giving a similarly high antibody response. Accordingly, two groups of 4 hamsters each were inoculated with 35 HA units of virus and kept under observation for 6 weeks.

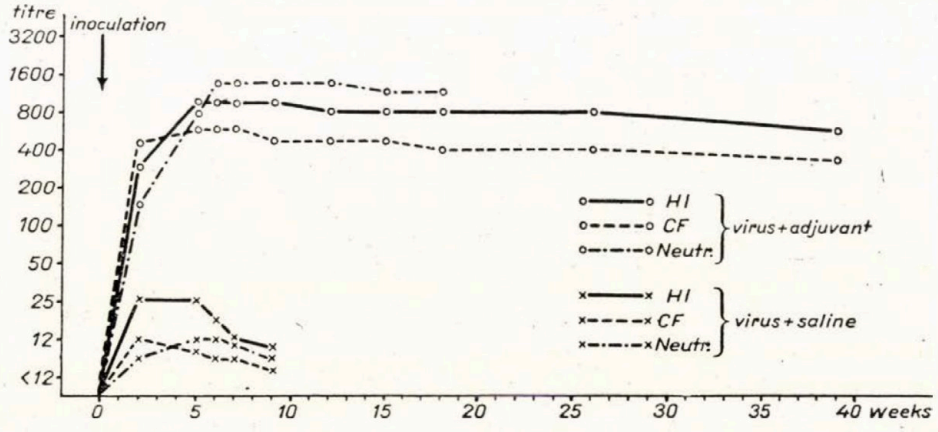

Fig. 1. Immune response in hamsters after inoculation of influenza A-prime (Lp) virus with oil adjuvant and in saline, respectively, each curve expressing the arithmetic mean of the antibody response for one group of six animals

Table I

Antibody response in hamsters after inoculation of 35 HA units of Lp virus

Mode of vaccination	Week after inoculation	Hamster serum							
		No. 1.		No. 2		No. 3		No. 4	
		HI	CF	HI	CF	HI	CF	HI	CF
Virus with adjuvant, 0,25 ml i. m.	2nd	<12	<12	<12	<12	300	300	<12	<12
	5th	25	25	75	75	400	600	<12	<12
	6th	25	25	75	75	400	600	<12	<12
Virus in saline 0,25 ml i. m.	2nd	<12	<12	<12	<12	<12	<12	<12	<12
	5th	<12	<12	<12	<12	<12	<12	<12	<12
	6th	<12	<12	<12	<12	<12	<12	<12	<12

It can be seen from Table I that no antibody was demonstrable in the sera of hamsters inoculated with vaccine without adjuvant, and that in the group given the adjuvant vaccine the antibody level is nil in one serum, low in two sera, and high in the fourth.

In a third experiment involving two groups of four hamsters each, each animal was given again 3500 HA units of virus, and blood was drawn from them every 4th day to compare the initial serum antibody levels after inoculation of virus with adjuvant and virus in saline.

Table II

Initial serum antibody levels in hamsters after inoculation of 3500 *HA units of Lp virus*

Mode of vaccination	Day after inoculation	Hamster serum							
		No. 7		No. 8		No. 9		No. 10	
		HI	CF	HI	CF	HI	CF	HI	CF
Virus with adjuvant, 0,25 ml i. m.	4th	<12	<12	<12	<12	<12	12	<12	<12
	8th	25	25	12	<12	12	12	<12	25
	12th	200	50	150	25	100	50	100	25
	16th	600	100	400	50	200	50	200	25
	36th	1600	400	1200	100	800	150	1200	100
Virus in saline, 0,25 ml i. m.	4th	<12	<12	12	<12	<12	<12	<12	<12
	8th	25	<12	50	<12	25	<12	25	12
	12th	40	<12	50	12	40	<12	25	12
	16th	40	<12	50	12	40	<12	25	12
	36th	40	<12	50	<12	25	<12	25	12

Table II shows that on the first 4 days following inoculation there was no immune response in either group, that on the 8th day the titres were of about the same level in both groups, and that while they reached their peak in the group without the adjuvant on the 12th day, in the group with the adjuvant they kept on rising, up to about 30 times the titres in the other group.

Immune response in rabbits. Two groups of 3 rabbits each were inoculated with influenza Lp virus with and without adjuvant, respectively. Three weeks later the injection was repeated. The arithmetic means of the antibody titres in the sera of the individual animals are presented in Fig. 2.

Fig. 2 clearly shows that in the sera of rabbits given the adjuvant the first sudden rise of the three titres occurred between the 4th and 6th week, and that the titres reached their peaks in 8 weeks after the second inoculation. 29 weeks later it was still possible to demonstrate these high immune titres. The situation was different with the sera of rabbits inoculated without adjuvant. There was no antibody response for 3 weeks after the first inoculation, but on the second injection there was a sudden rise which reached its peak in 1 or 3 weeks, to drop again abruptly. In the eighth week following the second injection, when in the other group the HI titre was at its highest, it was no longer possible to demonstrate with either test a titre rising above the non-specific pre-inoculation level. At that time the difference between the titres of the two groups was

at least a 24fold one. Another remarkable feature was the roughly identical height and chronological course of the HI and the virus-neutralising curves, while those of the CF titres run at a lower level but parallel with the others.

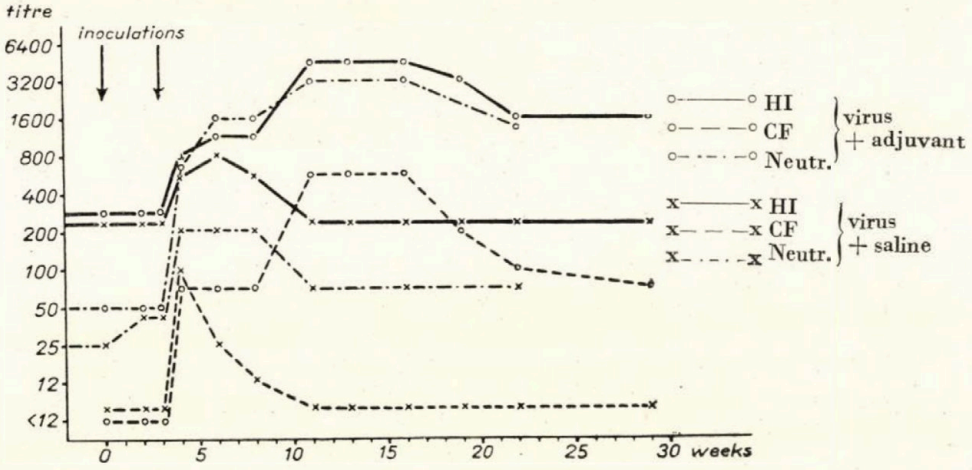

Fig. 2. Immune response in rabbits after inoculation of influenza A-prime (Lp) virus with oil adjuvant and in saline, respectively, each curve expressing the arithmetic mean of the antibody response of one group of three animals

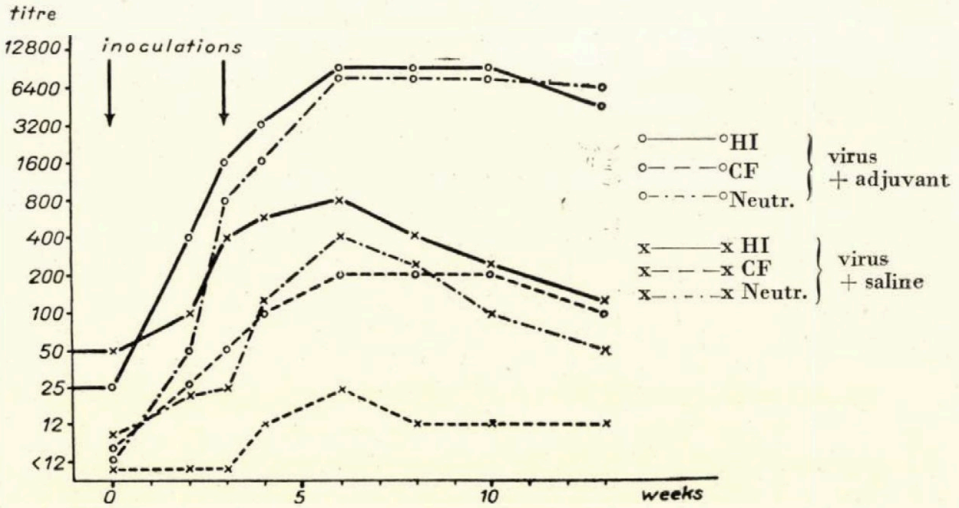

Fig. 3. Immune response in ferrets after inoculation of A-prime (Lp) virus with oil adjuvant and in saline, respectively, each curve expressing the arithmetic mean of the antibody response of one group of three animals

Immune response in ferrets. Twice 3 ferrets were inoculated with Lp virus on two occasions with 3 weeks between them. The results are presented in Fig. 3.

Fig. 3 reveals that in ferrets too the antibody level was higher if the animals had been given the virus with adjuvant than if without. The titres reached their peak in the sixth week after the first inoculation and were still high in the thirteenth week. The curves indicating the CF titres were much lower than the other two.

Immune response in rats. In experiments similar to the preceding ones rats were immunised. The titres obtained in the HI test were high, like in the hamster sera.

The immunogenic effect in hamsters of influenza type A and B strains. Prompted by experience gained in the preceding experiments, we inoculated hamsters with influenza type A, A-prime, and B strains. It was found that on mixing the virus with oil the immune response to one or two inoculations was considerably higher than that received after four inoculations with ten times the amount of virus without adjuvant. Only the Sweden 3/50 strain was an exception in this respect. Seven hamsters were inoculated with the oily emulsion of a preparation of this strain and revaccinated four weeks later with an other preparation of the same strain. In one of them only did the HI test show some very slight antibody response (1 : 100). The reason for this may be either that the two preparations of the Sweden 3/50 strain did not have a sufficiently strong immunogenic effect or that the antigenic capacity of the virus had been in some way influenced by the adjuvant. With a view to deciding this point, four weeks after the second inoculation, three hamsters were given a third injection consisting of the same virus preparation that had been used in the second inoculation but without adjuvant. To this the animals, which had been refractory to the injection with adjuvant, responded with a significant antibody level (1 : 100 to 1 : 400). A similar immune response developed in the other four hamsters which for a third inoculation were given Lp virus suspended in saline.

The antibody response to the third injection, one without adjuvant, permits the conclusion that the failure of the oily vaccinations was due to the application of the adjuvant.

On using the Sweden strain, the oil adjuvant failed to influence the antigenic capacity of the virus in rabbits and ferrets.

Local reaction to the inoculations. Minor or major lesions were invariably observed at the site of inoculation. In hamsters, the thigh was seen to swell the week after the injection ; in the second week, the infiltration gradually became circumscribed ; some weeks later, it was sometimes absorbed, but most usually one or more nodules, about the size of a bean, could be palpated. Microscopic inspection showed necrosis of muscular tissue with a great number of mononuclear cells around it. For these lesions probably the emulsifying agent, Arlacel A, was responsible. The local reaction was more marked in hamsters if inoculated with a 9 to 1 mixture of Bayol D and Arlacel A than if given

Bayol D oil in itself. In rabbits and ferrets the local lesions were more severe than in hamsters, and in some instances there was suppuration at the inoculation site.

Discussion

The use of the immune serum absorption test in the study of antigenic structures requires the application of high titre immune sera, since in them the differences between the strains can more reassuringly be determined. HIRST [24] produced them in rabbits, TAKÁTSY et al. [1, 2] in chicks, by inoculating the animals several times at various intervals with the respective virus. But this is a cumbersome method, which consumes much time and material. It has been found that by using an oil adjuvant one or two inoculations and a much smaller amount of virus suffice to produce immune sera of high titre. Hamsters were predominantly used in the pertaining experiments, because other investigations showed that they were the species most suited for the study of the antigenic structure of the influenza viruses [25]. The use of hamster immune serum has been rather uncommon, for only little serum can be obtained from one animal and, particularly in the case of the A-prime strains, the immune titres are low even after intranasal inoculation. The micromethod evolved by us for immune serum absorption tests [1, 2] and the application of an oil adjuvant render it possible to use hamster immune sera more widely when studying antigen structures.

Satisfactory results have also been obtained with oil adjuvants in the production of immune sera in the rabbit, the ferret, and the rat. It is remarkable that while in the hamster sera the HI, CF, and neutralisation titres were almost the same, in rabbit and ferret sera the curve for the CF titre ran a considerably lower course than the other two curves. On the other hand, the three kinds of curves were consistently parallel for each animal species.

SALK et al. [14, 15, 16] have demonstrated that in monkeys and human subjects A-prime strains induce a lower level of antibody production than A and B strains. But on the addition of an oil adjuvant a high level of antibody to the A-prime strains was obtained. Similar results were observed in the present experiments with hamsters. Only the Sweden 3/50 strain was an exception in this respect. The experimental results of ISAACS et al. [26] make it seem probable that the predominant antigen of strains in the Q phase, which is responsible for the immunogenic effect, lies deeper in the virus particle. We assume that if such strains, and the Sweden 3/50 strain is one of them, are inoculated into the animal organism, their dominant antigen cannot assert itself until after the organism has begun to decompose its superficial parts. It is not unlikely that the adjuvant effect consists in a retardation of this decomposition of the superficial parts wherefore the deeper placed predominant antigen is unable to induce antibody production.

Summary

Immune sera of high titre have been produced in hamsters, rabbits, ferrets, and rats by one or two inoculations with purified influenza virus mixed with an oil adjuvant. Sera of this kind are well suited for the study of antigenic structures, and permit of a more precise demonstration of the differences between strains. In hamster sera, the haemagglutination-inhibition, the complement-fixation, and the virus-neutralising titres were approximately the same; in the sera of the rabbit and the ferret, the CF titre was lower than were the other two titres.

On using the Q phase strain Sweden 3/50, the oil adjuvant failed to raise the antigenic capacity of the virus in rabbits, ferrets and hamsters.

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ПРОИЗВОДСТВО ИММУННЫХ СЫВОРОТОК ВЫСОКОГО ТИТРА В ОРГАНИЗМЕ ЭКСПЕРИМЕНТАЛЬНЫХ ЖИВОТНЫХ С ПОМОЩЬЮ ГРИППОЗНОЙ ВАКЦИНЫ, СМЕШАННОЙ С МАСЛЯНЫМ СТИМУЛЯТОРОМ

Я. ФЮРЕС

Р е з ю м е

Путем однократной или двукратной прививки очищенного гриппозного вируса, смешанного с масляным стимулятором, удалось получить обладающие сыворотки высокого титра от хомяков, кроликов, хорьков и крыс. Эти сыворотки более пригодны для исследования антигенной структуры вируса гриппа, с их помощью можно точнее определить различия между штаммами. В реакциях торможения гемагглютинации, связывания комплемента и нейтрализации титры сывороток хомяков были почти одинаковы. В реакции связывания комплемента титры сывороток кроликов и хорьков были на много ниже, чем в двух других реакциях.

В случае прививки хомяков, кроликов или хорьков шведским штаммом 3/50 A', находящихся в фазе Q, масляный стимулятор не повышает, а наоборот понижает иммуногенное действие вируса.

STUDIES ON THE EFFECTIVENESS OF AND REACTIONS TO ADSORBED BACILLARY TYPHOID VACCINE

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The antigens in anti-typhoid vaccines lack the world-wide uniformity of the antigens used in the prevention of diphtheria and tetanus [1]. In several countries bacteria killed without damaging the Vi antigen serve as antigens, in monovalent or in combined forms. On the basis of the fundamental research by LOVREKOVICH, RAUSS [2], and RAUSS [3] since 1937 the Boivin antigen (trichloracetic acid extract of typhoid bacilli) has been in use in this country, as the active ingredient of a precipitated vaccine. According to BOIVIN, MESROBEANU [4], RAUSS et al. [5], this extract contains all the immunologically important components of the bacterial cell. This claim seems to be amply substantiated by the fact that since the above vaccine had been introduced in Hungary, typhoid morbidity has been greatly decreasing. However, the reactions that develop after immunization, especially in repeatedly immunized individuals, tend to increase in severity. The complaints have been checked and re-checked by ERDŐS [6], who then confirmed that they were justified. Most of the subjects with complaints were adults, in whom extensive local, as well as highly febrile systemic reactions causing incapacity to work for a few days frequently occurred.

In view of the fact that severe reactions occurred mostly in repeatedly immunized individuals, it has been suggested that previous inoculations had sensitized them and that the local and systemic symptoms developed on subsequent inoculation were due to hypersensitivity. The literary reports on the sensitizing action of Salmonella and Shigella extracts seemed to confirm this suggestion. MORGAN could quantitatively titrate the antigen in the diethyleneglycol extract on grounds of the anaphylactic shock developing in guinea pigs which had been sensitized with bacterial immune serum and then incoculated with that extract [7]. DELAUNAY and PAGES sensitized guinea pigs with Boivin extract and subsequently elicited shock with the same extract [8]. Since in the above experiments sensitization to ingredients from the medium (peptone, agar) might have also occurred, we have examined the Boivin antigen extracted from bacteria grown on non-antigenic media and found it to be capable of sensitizing guinea pigs [9]. In further experiments it has been shown that in

guinea pigs sensitized with bacteria, or, passively, with antibacterial serum it is possible to induce anaphylactic shock with the Boivin extract, but also that the extract and its anti-serum can sensitize the animals to bacterial reinjection. The reactions produced by the extract were more severe than those produced by a dose of bacteria of the same N content [10].

The data in the literature indicate that the bacterial typhoid vaccine have good immunogenic properties. Bacteria, treated with acetone and dehydrated, are long known to be highly immunogenic, as it has been emphasized by HENDERSON [11], and recently by LANDY [12]. The bacterial vaccine thus prepared has been found to be superior in effect and stability to the heat-killed and phenolized one. LOVREKOVICH and RAUSS [22] have cited studies on adsorbed bacteria by VÁSÁRHELYI which, however, have to our best knowledge not been published. In 1946, MANN and SPINKA [13] immunized rabbits with adsorbed living typhoid bacilli, with good results. RÖHRER and DEHMEL [14] prepared a vaccine by adsorbing on aluminium hydroxide typhoid bacilli grown in broth. WOLTERS, FISCHOEDER and WEIDENMÜLLER [15] adsorbed from a liquid medium formol-killed typhoid bacteria.

On the basis of the above, we have adsorbed acetone-killed and dried typhoid bacteria on aluminium hydroxide or on alum and studied the properties of the vaccine thus prepared. The immunogenic potency of our vaccine was compared to that of the commercial RAUSS vaccine generally used in this country. In the sensitization experiments the question emerged whether the now usual human dose of the extract of 2500 million germs was not exaggerated and whether a decrease in the dose of antigen would not lessen the reaction without decreasing the antigenic response. The vaccine was therefore adjusted to contain the optimum amount of antigen in a reduced volume, *i. e* to include in 0,5 ml the amount of antigen to be used for human inoculation.

The following points were subjected to study:

(i) How are the immune response and the inoculation reaction affected by a reduction by 50 per cent of the now usual human dose of the extract vaccine?

(ii) What are the reactions to 0,5 ml of the adsorbed bacillary vaccine and what is the immune response of the organism to it?

Materials and methods

Strain. The Ty 2 strain of *S. typhi*, maintained by passage on Hartley agar and tested regularly by slide agglutination (O and Vi) and by virulence tests, was used. The LD 50 of the six-hour culture was 12 to 25 million germs without mucin, and 10 to 20 germs by the mucin technique.

Assay. The laboratory assay of vaccines was made according the routine procedure of the Department of the Vaccine Control, State Institute of Hygiene, *i. e.* by the standard immunizing dose — variable infective dose method. The protective value is understood to mean the ratio of LD_{50} for immunized mice to LD_{50} for control mice.

Rabbits, weighing 2,5 Kg, were immunized with a subcutaneous dose of 0,5 ml of vaccine. Blood was taken 4 weeks later. Mice were inoculated with half the human dose, *i . e.* with 0,5 ml Typhylax, or with 0,25 ml of adsorbed bacillary vaccine. Blood for agglutination tests was taken 4 or 6 weeks following inoculation.

For inducing passive immunity, sera were obtained by the ERDŐS technique [16] from each group inoculated with different vaccines or doses. In each group sera from 20 persons were pooled. Each serum was diluted in 5 steps, decreasing in geometrical progression. Twelve animals were inoculated with each dose ; a total of 60 white mice weighing 14 to 16 g was inoculated with a single pool of sera. The infective dose contained in 0,5 ml a six-hour peptonewater culture of Ty 2 organisms (as recommended by RAUSS) with 5 per cent Wilson granular mucin, 1 in 4 [17]. The mice were infected 1 hour after inoculation with serum. In the evaluation — the 48-hour deaths were considered with each titration, cultures were made of the heart blood from mice killed by each of five different dilutions. The live microbial count of the infecting suspension was estimated by dripping 0,1 ml of the suspension on a dried agar plate.

Preparation of vaccine. Strain Ty 2 was cultured for 36 hours at 37 °C in the DOLE dialysed medium [18], as modified by us [9], in the presence of bromothymol blue, adding N/10 NaOH, and shaking at intervals. After controlling the pure culture and the colonies for agglutinability, the culture was centrifuged under sterile conditions, suspended in sterile acetone, repeatedly washed, passed through a Jena G_5 filter and dehydrated with acetone. Tests for bacterial death and for eventual contamination were carried out in each step. The removal of acetone from dried bacteria was carried out by passing sterile air, in a simple apparatus of our own design. The bacterial powder thus obtained was readily homogenizable and suspendable and lost none of its immunogenicity after 1 year at room temperature.

The bacteria thus treated were readily adsorbed on an alum precipitate in saline medium. Aluminium hydroxide (4 to 5 mg/ml) adsorbs bacteria permanently as it was determined after 1 year of storage. In establishing the optimum for alum precipitation we employed the method described by LOVREKOVICH and RAUSS. Adsorption was controlled by centrifuging at 1000 r. p. m. for 2 minutes.

The adsorbed extract vaccine used for comparison was a preparation from the "Human" Institute for Vaccines Production and Research. Batch Nos. 14, 15, 16, 17 and 18 all containing the Boivin type antigen precipitated with alum. The dose of this vaccine is 1,0 ml. The adsorbed bacillary vaccine contained in each ml 2000 million germs adsorbed on alum precipitate. Of this vaccine, 0,5 ml were injected into the upper arm.

Technique of inoculation. After carrying out the control tests prescribed by law, a few volunteers were inoculated with the adsorbed bacillary vaccine. After having recorded their mild reactions to inoculation, the vaccine was tried out on a larger scale, by inoculating human subjects of various ages. Since our purpose was to observe the reaction of subjects who before had not been vaccinated, the test inoculations were made in children 6 to 12 years old.

Results

The data of the standard control tests for one of the commonly used vaccines and for our experimental vaccine are presented in Table I. Both vaccines have met all the requirements they were tested for. The experimental vaccine contained about double the amount of nitrogen than the commercial vaccine, a fact of some importance with respect to inoculation reactions, considering an eventual sensitization.

The passive immunisation values given in Table I represent the results obtained in tests on pooled sera from inoculated children. The values stand for the potency ratio (adsorbed extract vaccine = 100), as calculated by probit analysis.

In Table II the local reactions occurring after a dose of 0,5 ml of the different batches of adsorbed extract vaccine are compared with those produced

by 0,5 ml of the adsorbed bacillary vaccine. The total number of children inoculated with the experimental vaccine and subjected to subsequent control was 186 ; of these infiltration accompanied by erythema more than 5 cm in diameter occurred in 18 (10 per cent). It is remarkable that the incidence of local reactions was higher with each kind of adsorbed extract tested than with

Table I

Results of control tests with adsorbed bacillary vaccine and commercial adsorbed extract vaccine

Property tested	Adsorbed extract vaccine No. 14.	Adsorbed bacillary vaccine No. 19
Aluminium content, mg/ml	2,3	2,55
pH	5,6	6,2
N content, mg/human dose	0,0194	0,0415
Protective value LD 50	5,3	6,2
Rabbit serum.		
Agglutination titre		
after 4 weeks	1 : 1600	1 : 800
Mouse serum		
Agglutination titre		
after 4 weeks	1 : 400	1 : 400
after 6 weeks	1 : 100	1 : 200
Passive protective value of serum	100	$181 < 257 < 382$

Table II

Local reactions to 0,5 ml doses of vaccines
Szekszárd, schoolchildren, March, 1955

Vaccine	Total number of inoculated persons	Of these : local reactions	
		number	per cent
Adsorbed extract vaccine			
No. 14	57	10	18
No. 16	41	5	12
No. 17	42	7	17
No. 18	35	10	29
Total	175	32	18
Adsorbed bacillary vaccine	186	18	10

Statistical values for difference :

$\chi^2 = 4{,}90 \qquad P < 5\%$

the experimental vaccine. An overall evaluation revealed that out of a total of 175 inoculated children 32 (18 per cent) developed such local reactions. This incidence is about double that observed with the experimental vaccine. The difference was statistically significant. This means that local reactions were less frequent after the experimental vaccine than after the commercial one.

Some of the reactions can of course be estimated only from the complaints of the inoculated person. For example, local tenderness, head-ache, nausea cannot be evaluated as objectively as it is required with statistical analysis. The same applies to fever, in which case it would be possible to follow the behaviour of the temperature, but in practice this is almost unfeasable. The data

Table III

Comparison of the reactions caused by two different typhoid vaccines

Vaccine	Total number of inoculated persons	Systemic and local reactions	Systemic reactions only	Systemic reactions Total	
				number	per cent
Adsorbed extract vaccine					
No. 14	57	7	27	34	60
No. 16	41	3	15	18	44
No. 17	42	5	21	26	62
No. 18	35	5	11	16	46
Total	175	20	74	94	54
Adsorbed bacillary vaccine ..	186	7	45	52	28

Statistical values for difference :

$\chi^2 = 23{,}78 \qquad P < 0{,}1\%$

in Table III should therefore be regarded with some reservation. The reactions mentioned in the history have been divided into a group containing individuals with apparent local changes and systemic reaction, and in another group those with systemic complaints but no local change. The latter group surpassed the former, both numerically and in percentage, with both types of vaccine. Table III shows that the incidence of systemic reactions produced by the experimental vaccine was lower than that produced by the extract vaccine.

One group of school-children was inoculated, instead of 0,5 ml, with 1,0 ml of the control adsorbed extract vaccine. This group then supplied information to show whether one might expect a mitigation of the reaction from a reduction from 1,0 to 0,5 ml of the single inocula. The results are presented in Table IV. As it can be seen, the 1,0 ml dose caused a local reaction more often than the reduced dose. An analysis of the results reveals that the incidence of local

reactions was 24 per cent after the 1,0 ml dose, but only 18 per cent after the 0,5 ml dose. This divergence is less than that in the former comparison, and calculations showed that statistically it was not significant. Thus, it cannot be stated with certainty that a reduction by 50 per cent of the volume of the dose would always lessen the inoculation reaction. Similar findings have been reported by ERDŐS [6], for individuals of different ages inoculated in 1953.

Table IV

Local reactions to 1,0 *ml dose, as compared to those to* 0,5 *ml of adsorbed extract vaccine*
Szekszárd schoolchildren, 1955.

Vaccine and dose	Total number of inoculated persons	Of these : Local reactions	
		number	per cent
1,0 ml of adsorbed extract vaccine			
No. 14	155	33	21
No. 16	129	28	22
No. 17	151	41	27
No. 18	158	40	25
Total	593	142	24
0,5 ml dose	175	32	18

Statistical values for difference :

$\chi^2 = 2{,}16$ $\quad P < 16\%$

The potency of the vaccines was determined by the passive immunity test described in the section on methods, carried out on pooled sera from each

Table V

Protective value of different volumes of human serum, as determined in mice infected with an identical number of germs
Schoolchildren. Blood taken 7 weeks after inoculation

Volume of serum ml	Adsorbed bacillary vaccine 0,5 ml	Adsorbed extract vaccine	
		0,5 ml	1,0 ml
	Of the 12 mice per dose survived		
0,003125	0	0	0
0,00625	3	0	0
0,0125	11	1	3
0,025	11	9	4
0,05	12	11	10

The mice had been infected with about 500 LD 50.

group of inoculated individuals. The results are shown in Table V. It will be seen at a glance that the experimental vaccine was superior to the commercial ones. The experimental vaccine induced immunity in a dose as low as 0,00625 ml, while the sera from individuals inoculated with the other two vaccines were only slightly protective in a dose as high as 0,0125 ml. Another way for obtaining preliminary information is to consider the total number of survivors. Of the

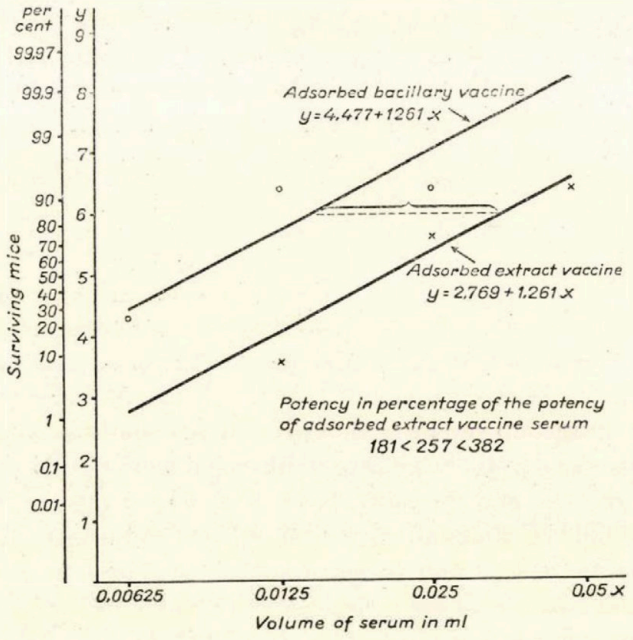

Fig. 1. Comparative presentation of the potency of the adsorbed bacillary vaccine and of the adsorbed extract vaccine sera. Values obtained : Adsorbed bacillary vaccine : ∘ Adsorbed extract vaccine : × Potency in percentage of the potency of adsorbed extract vaccine serum. Volume of serum in ml.

mice inoculated with sera from individuals inoculated with the experimental vaccine, 37 survived, as compared to the 21 survivors after 0,5 ml of adsorbed extract vaccine and the 17 survivors after the 1,0 ml series.

The effectiveness of the inoculation is shown also by the fact that the protective value of a pool of sera from 20 children before inoculation was higher than 0,05 ml. The results of statistical evaluation by probit analysis, are shown in Fig. 1. According to the calculations for error, the experimental values could be approached within the permissible limits of error with direct lines and at the same time the parallelism of the two lines was also confirmable. Accordingly, it was possible to determine the relative potency of the two sera. As established 7 weeks after inoculation with 0,5 ml of vaccine, the protection afforded by the

experimental vaccine was about 2½ times higher (exactly 257 per cent) than that reached after the adsorbed extract vaccine. According to the limits of error, the protective power of the serum of the experimental vaccine was not less than 180 per cent of the protective capacity of the other serum (95 per cent probability).

As it has been mentioned, the serum obtained from persons inoculated with 1,0 ml of adsorbed extract vaccine proved to be less protective than the serum from individuals inoculated with 0,5 ml doses. Probit analysis has shown that the protective value of the serum of the 0,5 ml inoculations was 23 per cent higher than that of the 1,0 ml dose. It had, therefore, to be assumed that in this case the inoculations with higher dose were less effective.

Discussion

A survey of the literature has shown that there are only scarce data on the numerical evaluation and analysis of reactions following inoculation with bacillary typhoid vaccines. PÁTER compared the RAUSS vaccine to the mercury bichloride-killed bacterial vaccine and found that the former produced milder reactions [19]. LUIPPOLD reported that after 3 inoculations with the T.A.B. vaccine local reactions in the form of an infiltration more than 5 cm in diameter occurred in 91 per cent, and systemic reactions in 56 per cent, of the cases [20]. FELIX observed mild reactions after his alcohol-killed and preserved vaccine [21].

According to reports and textbooks, the more or less severe reactions that develop following inoculation might be correlated with sensitizing stimuli affecting the organism before inoculation. SDRODOVSKY [22] and ZINSSER [23] are of the opinion that on repeated inoculation sensitization develops to the antigen of the typhoid bacillus. Our experiments with bacteria (or their extracts) from non-antigenic media showed that guinea pigs could actually be sensitized, and have thus lent support to the above view. The increase in the severity of the reaction after inoculation observed mainly in the adult and repeatedly inoculated population may be ascribed to such a sensitization (BOZSÓKY, HEGYESSY, UHL, [24]).

The question is, therefore, whether milder reactions are to be expected to occur after the adsorbed bacillary vaccine, which contains twice as much nitrogen per dose as the commercial vaccine.

We believe that one of the principal causes of the slighter inoculation reactions is the difference in the dispersion of antigen. According to yet unpublished experiments, guinea pigs sensitized with bacterial extracts developed more regularly and markedly an anaphylactic shock after the extract, than after the bacterial suspension containing the same amount of nitrogen as the extract. It is thought that the inoculated bacterial cell (which may be considered

a major unit) gives off the antigens involved in sensitization at a slower rate so that the eventually developing allergic reaction will be less severe. In support of this view is the observation by WILSON and MILES [25], that it is more difficult to induce sensitization with bacillary antigen, than with horse serum or egg albumin. According to BRONFENBRENNER [26], both the complicated arrangement of bacterial antigens and an aggregation of antigenic parts are to be held responsible for the development of the different types of hypersensitization.

It would be incorrect to blame solely the hypersensitivity factor for the reactions after inoculation, since individuals who had never been in contact with typhoid antigen also develop reactions following inoculation. The extracts and bacteria are well-known to kill mice, and this property is called toxicity. DARVAS and UJHELYI [27] purified with cold methanol the Boivin extract and obtained a substance which was less toxic, but whose antigenicity remained unaffected. However, reactions occurring after the use of the precipitated vaccine prepared from this less toxic substance were not significantly milder.

It is remarkable, and needs therefore an explanation that the serum from individuals inoculated with larger doses of commercial vaccine had somewhat less mouse protective potency than the serum obtained after inoculations with a smaller dose. The importance of the quantity of antigen used for inoculating man or experimental animals has been stressed in numerous reports. In their experiments with pneumococcal polysaccharide, ZOZAYA and CLARK [28], WADSWORTH and BROWN [29], as well as FELTON [30], have pointed out that excessive doses of antigen do not protect mice against superinfection ; on the contrary, the sensitivity is further increased. These animals will respond with a reduced antibody production to non-paralysing doses of antigen given later in their life. FELTON called this phenomenon an "immunological paralysis" and in later experiments found it to be explicable by a prolonged storage of the polysaccharide in the tissues of the animal. Since the Boivin extract used for immunising man contains 30 to 50 per cent polysaccharide, a process similar to that described above may be conceived to take place.

Summary

We have prepared a vaccine from acetone-killed, dehydrated and adsorbed typhoid bacteria. In comparative human inoculation tests against the adsorbed Boivin extract vaccine (the commercial product "Typhylax") the following have been established :

1. A reduction from 1,0 to 0,5 ml of the single dose of Typhylax did not result in a significant mitigation of the reactions occurring after inoculation. The effectiveness of immunisation did not diminish ; on the contrary, it seemed to provide a better protection than that afforded by the 1,0 ml dose.

2. The adsorbed bacillary vaccine produced significantly less post-inoculation reactions than did the commercial vaccine. Sera from individuals inoculated with the former vaccine had an estimated mouse protective power about $2\frac{1}{2}$ times higher than that demonstrable in sera after inoculation with adsorbed extract vaccine.

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ИССЛЕДОВАНИЕ ЭФФЕКТИВНОСТИ АДСОРБИРОВАННОЙ БАЦИЛЛЯРНОЙ ТИФОЗНОЙ ВАКЦИНЫ И ПРИВИВОЧНЫХ РЕАКЦИЙ

ДЬ. ТОЛЬНАИ и ДЬ. БАРШИ

Резюме

Авторы изготовили прививочный материал из убитых ацетоном и дегидрированных, адсорбированных тифозных бактерий. Этот прививочный материал — при вакцинации людей — сравнивали с находящимся в обороте прививочным материалом, содержащим экстракт Буавена и установили следующее:

1.) Сокращение дозы Тифилакса с 1,0 мл. до 0,5 мл. не вело за собой значительного понижения пропорции прививочных реакций. Эффективность прививок не понижалась, а вероятно была лучше, чем после прививок в дозе 1,0 мл.

2. Адсорбированная бациллярная вакцина вызывала значительно меньше прививочных реакций, чем находящийся в обороте прививочный материал. Защитная ценность для мышей сыворотки привитых лиц, вероятно, более чем в два с половиной раза выше защитной ценности для мышей, обнаруживаемой в сыворотке привитых Тифилаксом.

ELECTRONMICROSCOPIC STUDIES OF THE FILTRATES OF AGED SALMONELLA CULTURES

By

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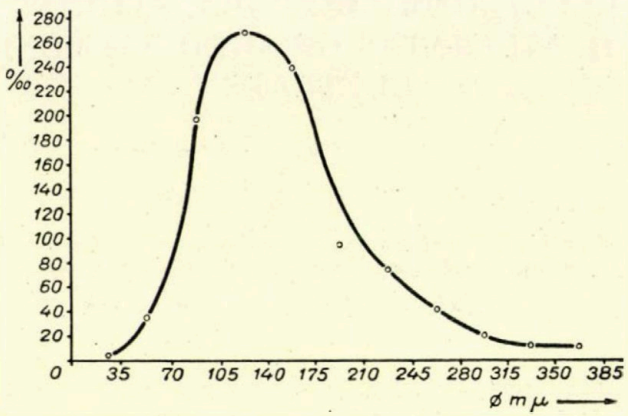
The filterable forms of bacteria and the process of regeneration starting out from filterable forms have been much discussed in recent literature. Filterable forms may be obtained from normal bacteria by a variety of methods, for example by chemical treatment [11, 12], by exposure to immune serum [10], phage [18], antibiotics [5, 6], by ultrasound treatment [11], by mechanical destruction [13], etc. One of us has carried out successful regeneration experiments with filterable forms obtained by mechanical treatment of cultures of *Salmonella enteritidis var.* Danysz as well as by aging such cultures [14]. In the course of this work evidence has been obtained corroborating the data in the literature [5, 6, 7], according to which under appropriate conditions it is through granular, coccal and cocco-bacillary forms that the Salmonellae reach complete regeneration (Figs. 2—5). Absolutely irregular, bizarre forms may also arise in the course of regeneration (Fig. 4). There are, however, few data concerning the size and detailed morphology of filterable forms and, as it will be pointed out later, most of these data concern the L forms of bacteria and the smallest reproductive units of pleuropneumonia-like organisms. It has therefore been thought that studies under the electronmicroscope of such filtrates of aged Salmonella cultures, from which complete regeneration of Salmonella could be achieved, will supply interesting and hitherto lacking evidence.

Materials and methods

A strain of *Salmonella enteritidis var.* Danysz was kept in 10 per cent horse serum broth at 37° C for a few days, and subsequently at room temperature for 7 to 45 days. The cultures thus aged were passed through Seitz „S" and „EK" filters, and centrifuged at 40 000 r. p. m. for 1 hour in a Phywe ultracentrifuge. The sediment was suspended in physiological saline and a drop of suspension was transferred to a micro-screen for electronmicroscopic examination. After drying and elution of salt, the preparation was shaded with gold. A TTC electronmicroscope, operated at 40 kV, was used.

Results

In aged cultures we have found great numbers of bacteria, whose cytoplasm was granular in structure, in contrast with the homogeneous cytoplasm of the logarithmic phase dividing forms (Fig. 1). The granules were spherical



Graph. 1. Distribution of filterable forms of *Salmonella enteritidis var.* Danysz, in the function of diameter

in shape and about 200 mμ in diameter (Fig. 2). When the bacterium disintegrates, these granules may enter the medium, and when cultures are filtered, they may pass over into the filtrate by virtue of their minute size and, under favourable conditions, may serve as the starting point of regeneration.

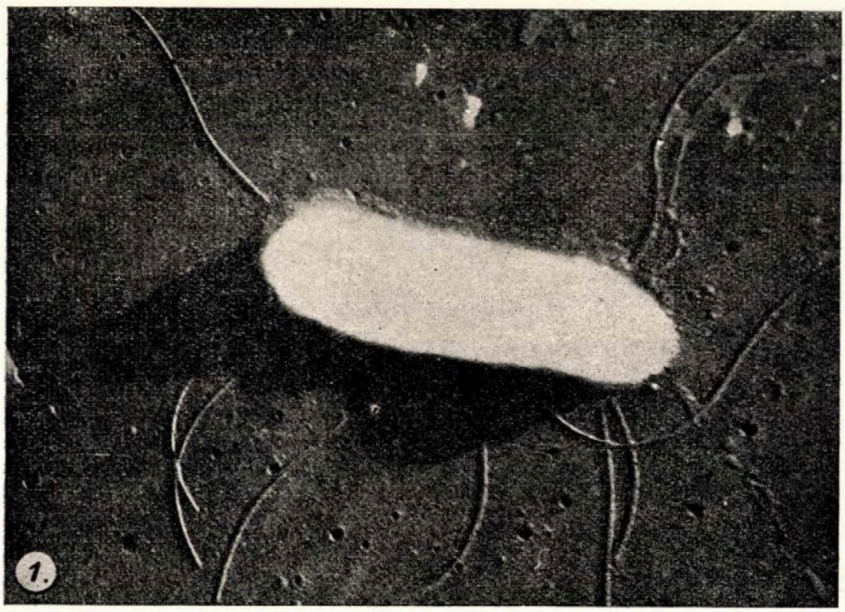


Fig. 1. Normal, young bacterium from 6-hour broth culture. × 30 000

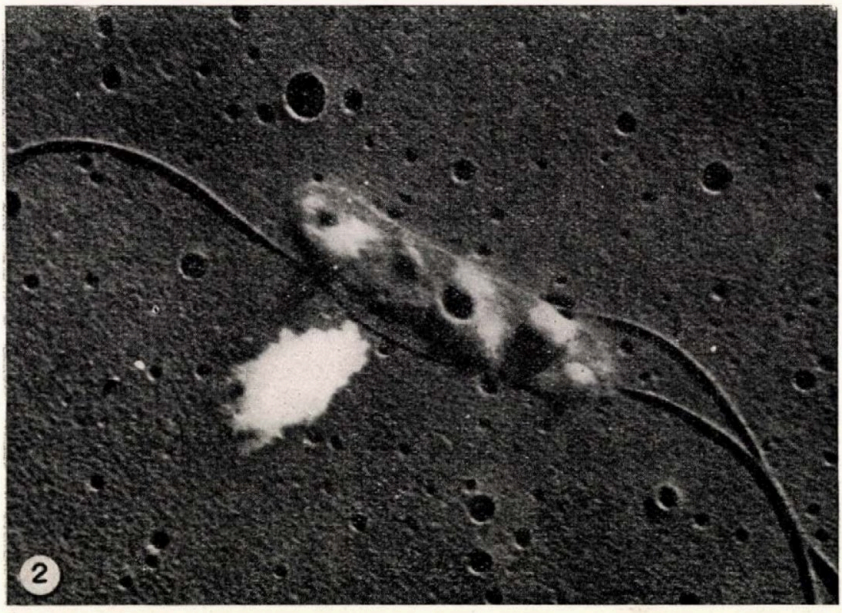


Fig. 2. Old bacterium, showing granular structure, from 45-day culture. × 30 000

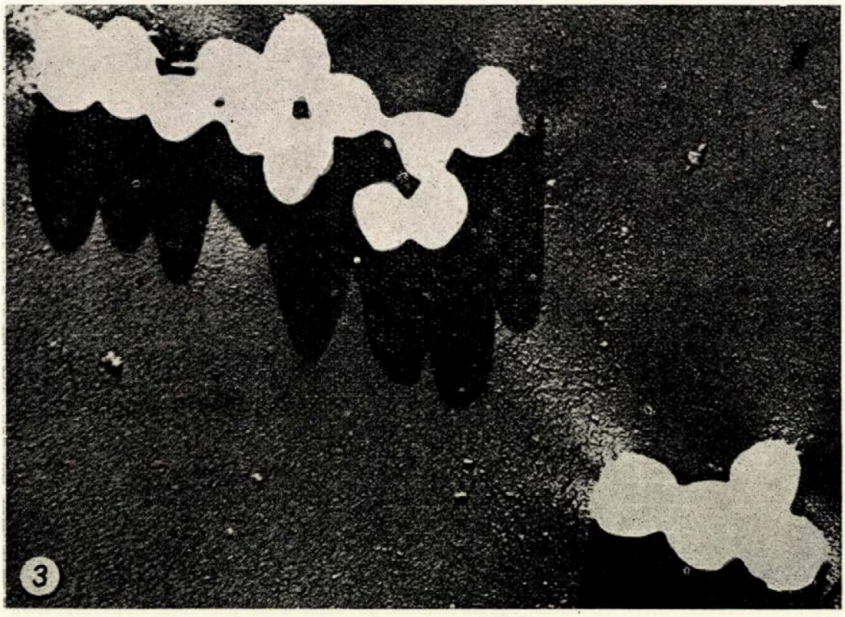


Fig. 3. Regeneration of cocci from filtrate. × 12 000

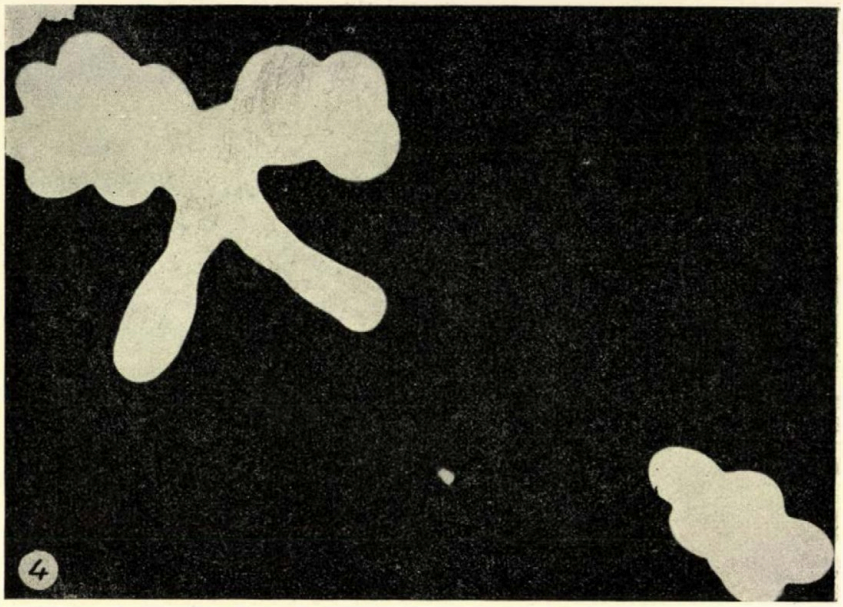


Fig. 4. Bizarre coccal forms from regenerating filtrate. × 12 000

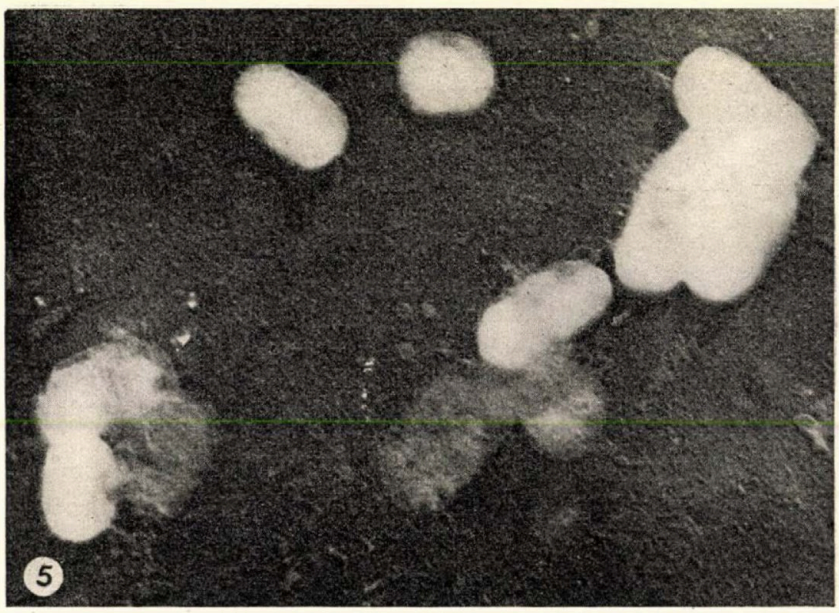


Fig. 5. Cocco-bacillary forms from regenerating filtrate. × 15 000

Fig. 6. Particles from the filtrate of a 45-day culture, concentrated by centrifugation. × 14 500

Fig. 7. Aggregates from serum-broth medium. × 17 000

The material worked up remained sterile during filtration and centrifugation and, as it has been proved by sterility tests, intact Salmonellae did not pass the filter.

Electronmicroscopy revealed the presence in the filtrate, concentrated by centrifugation, of a great number of particles, more or less regularly spherical

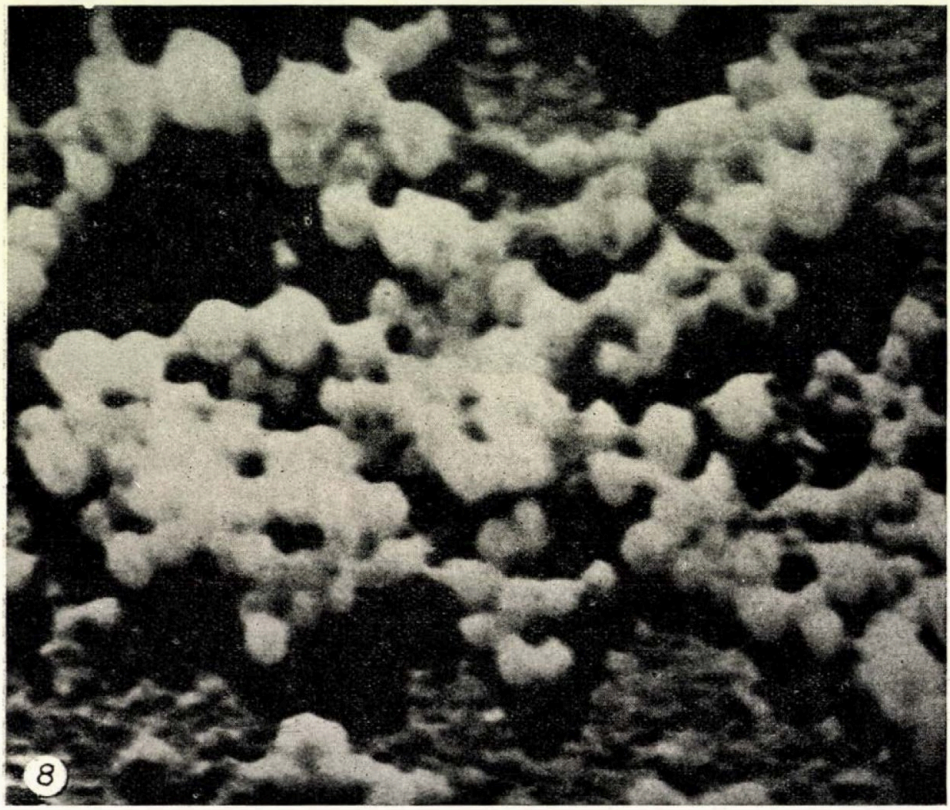


Fig. 8. High-power view of an area in Fig. 6. The filterable forms appear as well-defined spherical structures. × 55 000

in shape (Fig. 6) and definitely distinct from the amorphous substance of the serum-broth component in the filtrate (Fig. 7). They bore some resemblance to the transparent forms described by BRINGMANN [1]. Some of the particles contained a dark material but it could not be decided on the basis of the photographs whether or not these forms possessed some primitive cellular structure.

As the size distribution curve (Graph. 1) determined by measuring 1000 particles reveals, most of the particles (87,6 per cent) were 70 to 245 mμ

in diameter, and within that range most of them fell between 70 to 175 mμ.

These data roughly agree with those published in the literature. KALINA [7], TULASNE [16, 17], JUHÁSZ [5] estimated the size of filterable forms to vary from 200 to 300 mμ, while FOSHAY LEE and HESSELBROCK [3] to between 300 to 350 mμ. KLIENEBERGER-NOBEL [8, 9] in her filtration studies on the L forms of *S. moniliformis* found that a gradocol membrane of 350 mμ pore diameter retained all filterable forms, and estimated the particle size to vary from 175 to 250 mμ, according to the ELFORD formula. In electronmicroscopic studies on PPLO, SMITH, MUDD and HILLIER [15] discovered intracellular granules 100 to 180 mμ in diameter. FREUNDT [2] studied human PPLO under the electronmicroscope and demonstrated chains consisting of elementary bodies of about 180 to 250 mμ in size.

Comparing the data in the literature with those of our own, as regards the size of filterable forms we accept the standpoint of KALINA [7] that "the filterable forms probably vary in size, from forms visible under the electronmicroscope to those measurable by light microscopy". It has, however, to be added that as far as regeneration is concerned, the filterable forms cannot be considered to be uniform, since those below a certain order of magnitude will not regenerate even under optimal conditions. It may also be assumed that regenerability varies from species to species.

Summary

Filtrates of aged cultures of *Salmonella enteritidis var.* Danysz have been studied under the electronmicroscope. When under suitable cultural conditions, these filtrates had been shown to yield completely regenerated Salmonellae. In the sediment of the ultracentrifuged sample large numbers of spherical, partly transparent particles (of which some contained a dark substance) have been found. By measuring 1000 particles, it could be shown that most of them (87,6 per cent) varied from 70 to 245 mμ in size. Within that size range, the majority of particles fell within the limits of 70 to 175 mμ. Granules of similar shape and size have been seen within aged bacteria. On the basis of the evidence obtained it may be assumed that the aged bacteria disintegrate and release their granules. These granules may then serve as the starting point of subsequent regeneration.

Acknowledgement

We are indebted to Mrs M. D. EGYESSY and Miss M. MERSVA for their cooperation in this work.

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ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ ФИЛЬТРАТОВ УСТАРЕЛЫХ КУЛЬТУР SALMONELLA

И. НАС и Б. ЛОВАШ

Резюме

Авторы исследовали электронным микроскопом обогащенные ультрацентрифугой фильтраты устарелых культур Salmonella enteritidis var. Danysz, исходя из которых при благоприятных условиях удалось достичь полной регенерации культур Salmonella. В осадке ультрацентрифугированного вещества авторы обнаружили большое количество шарообразных, частью прозрачных частиц, показывающих темное содержимое. После измерения дивметра тысячи частиц определили, что размер 87,6% измеренных частиц колеблется в пределах 70–245 mμ. В том числе большинство частиц имело величину в 70–175 mμ. Внутри устарелых бактерий были видимы частицы подобной формы и величины. На основании исследований авторы предполагают, что устарелые бактерии распадаются, и частицы их освобождаются. Эти частицы в дальнейшем могут служить исходной точкой регенерации.

ZUSAMMENHANG ZWISCHEN KONZENTRATION UND WIRKUNG DER DESINFIZIENZIEN

Von

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(Eingegangen am 21. Januar 1956)

In einer vorigen Mitteilung [1], in der wir den Temperaturquotienten einiger Desinfizienzien bestimmt hatten, stellten wir fest, dass die moderne Laboratoriumskontrolle der Desinfizienzien mit der Bestimmung von erheblich mehr charakteristischen Eigenschaften verknüpft werden muss, als dies bisher geschah. Im Rahmen dieser Bestrebungen beschäftigen wir uns in der vorliegenden Arbeit mit den verschiedenen Konzentrationen einhergehenden Veränderungen der Desinfektionswirkung. Diese werden in der Literatur als Konzentrationsexponent bezeichnet.

Aus der Literatur verweisen wir nur auf die bahnbrechende Arbeit von CHICK [2], da uns sonst nur sehr wenige Angaben zur Verfügung standen. CHICK wies schon 1908 darauf hin, dass zwischen der Phenol-Konzentration und der zur Erreichung der bakteriziden Wirkung erforderlichen Zeit eine bestimmte, nicht einfach umgekehrte Proportionalität besteht. Dies bedeutet, dass sich z. B. bei der doppelten Phenol-Konzentration die Einwirkungszeit nicht, wie zu erwarten wäre, auf die Hälfte, sondern in grösserem Ausmass vermindert. Später wurde dieser Zusammenhang auf Grund der CHICKschen Angaben von WATSON [3] auch mathematisch bestimmt.

Methodik

Unter Berücksichtigung der Faktoren, welche in der Desinfektionswirkung eine wichtige Rolle spielen, standardisierten wir bei unseren Versuchen die Bakterienmenge und Temperatur. Wir verwendeten zu dem entsprechend verdünnten Desinfiziens 0,5 ml einer 18—20stündigen Bouillonkultur des auf übliche Weise vorbereiteten *Salmonella typhi*-Stammes. Die Umimpfungen nahmen wir mit der gebräuchlichen Öse von 4 mm Durchmesser in Röhrchen vor, die etwa 12 ml Fleischextraktbouillon enthielten. Die Subkulturen wurden 48 Stunden bei 37° C inkubiert. Die Versuche führten wir bei genau 20° C im Wasserbad durch. Es wurden folgende Desinfizienzien untersucht: Formalin, Phenol, $HgCl_2$, Neomagnol, Nitrogenol (Hexadezylpyridiniumbromid) und Ryfen (Quecksilberphenylborat). Die bei den Versuchen angewandten Konzentrationen der Desinfizienzien wurden auf Grund der Erfahrungen mehrerer Vorversuche gewählt. Bei jedem Desinfiziens suchten wir unter mehreren verschiedenen Konzentrationen diejenige Wirkungsdauer, bei der obige Bakterienmenge bei 20° C mit Sicherheit getötet wird. Zu diesem Zweck gingen wir in der Weise vor, dass wir die Umimpfungen aus dem Gemisch Desinfiziens + Bakterium anfangs 2½ minütlich, von 30 Minuten bis zu 1 Stunde 5minütlich, nach 1 Stunde bis zu 2 Stunden 10minütlich vornahmen. Die Konzentration von jedem Desinfiziens wurde in 10 Parallelversuchen untersucht. Zur statistischen Verarbeitung wurden die Resultate der aus den Paralleluntersuchungen gewonnenen erfolgreichen Wirkungszeiten verwendet.

Ergebnisse

Die untersuchten Konzentrationen der Desinfizienzien und die geometrischen Durchschnittswerte der bei den einzelnen Konzentrationen beobachteten erfolgreichen Einwirkungszeiten sind aus Tab. I ersichtlich.

Tabelle I

Durchschnittliche erfolgreiche Einwirkungszeit der untersuchten Konzentrationen einiger Desinfizienzien (S. typhi)

Desinfiziens	Untersuchte Konzentration	Erfolgreiche durchschnittliche Einwirkungszeit in Min.
Formalin	1 : 216	50,0
	1 : 144	27,3
	1 : 96	18,6
Nitrogenol	1 : 33750	57,3
	1 : 22500	29,1
	1 : 15000	17,3
	1 : 10000	10,0
Ryfen	1 : 67500	88,9
	1 : 45000	47,1
	1 : 30000	23,5
	1 : 20000	9,1
$HgCl_2$	1 : 125000	14,1
	1 : 100000	9,4
	1 : 80000	5,9
Neomagnol	1 : 10646	18,7
	1 : 9258	12,7
	1 : 8050	8,5
	1 : 7000	6,1
Phenol	1 : 110	23,0
	1 : 100	16,2
	1 : 90	10,0
	1 : 80	6,8

Ausser bei Phenol wurden die Konzentrationen nach geometrischer Progression zusammengestellt. Bei Formalin, Ryfen und Nitrogenol wandten wir als Verdünnungsmassstab 1,5, bei $HgCl_2$ 1,25, bei Neomagnol 1,15 an. Im Hinblick darauf, dass wir bei den Konzentrationen der verschiedenen Desin-

fizienzien unterschiedliche Verdünnungen benutzten, lassen sich die geometrischen Durchschnittswerte der erfolgreichen Einwirkungszeiten bei den verschiedenen Desinfizienzien nicht miteinander vergleichen. Soviel können wir jedoch auf Grund der Angaben in Tab. I für jedes Desinfiziens feststellen, dass zwischen Konzentration und erfolgreicher Einwirkungszeit ein entschiedener Zusammenhang besteht. Desinfizienslösungen mit höherer Konzentration weisen in jedem Fall eine kürzere Einwirkungszeit auf als die stärkeren Verdünnungen.

Auf Grund der Resultate von CHICK stellte WATSON fest, dass die erfolgreiche Einwirkungszeit zu der je Desinfektionsmittel variierenden Potenz n der Konzentration des Desinfiziens im umgekehrten Verhältnis steht. Dementsprechend ist das Produkt der erfolgreichen Einwirkungszeit und der Potenz n der Konzentration eine konstante Zahl:

$$t \,.\, C^n = a,$$

wo t die erfolgreiche Einwirkungszeit, C die Konzentration und a einen konstanten Wert bedeutet. Logarithmieren wir vorstehende Gleichung, gewinnen wir auf $\log t$ gelöst:

$$\log t = \log a - n \log C$$

wo also $\log t$ als lineare Funktion von $\log C$ auftritt, deren unabhängige Variable $\log C$, Richtungstangente n und Konstante $\log a$ ist. Auf Grund obiger Zussammenhänge konnte die statistische Aufarbeitung der Resultate ohne weiteres vorgenommen werden. Die untersuchten Konzentrationen sowie die zu den einzelnen Konzentrationen gehörenden, in Minuten ausgedrückten erfolgreichen Einwirkungszeiten mussten logarithmiert werden. Den gewonnenen Resultaten näherten wir uns unter Anwendung der Methode der kleinsten Quadrate mit einer Regressionsgeraden, deren Formel wir errechneten. Zur Kontrolle untersuchten wir in jedem Fall statistisch, ob man die beobachteten Angaben innerhalb der vorgeschriebenen Fehlergrenzen mit einer geraden Linie erreichen kann und ob wir als Richtungstangente der Regressionsgeraden bewertbare Zahlen gewonnen hatten. Die Nachprüfung ergab in allen Fällen ein zufriedenstellendes Resultat, d. h. wir konnten zwischen der Konzentration der Desinfizienzien und ihrer Einwirkungszeit den gleichen Zusammenhang feststellen, der schon von CHICK und WATSON beschrieben wurde.

Die durchgeführte Berechnung bzw. ihre Resultate wollen wir an einem der Desinfektionsmittel, dem Neomagnol, etwas ausführlicher demonstrieren (Abb. 1).

Auf der rechten Seite der Abb. 1 veranschaulichen wir den Zusammenhang zwischen den Logarithmen der Konzentrationen und den Logarithmen der Einwirkungszeiten. Die geometrischen Durchschnittswerte der bei den einzelnen Konzentrationen gewonnenen erfolgreichen Einwirkungszeiten sind mit einem

kleinen Kreis bezeichnet. Aus der Darstellung geht hervor, dass diese beobachteten Resultate mit einer geraden Linie gut erreicht werden können und dass zwischen den Logarithmen der Konzantrationen und den Logarithmen der erfolgreichen Einwirkungszeiten ein geradliniger Zusammenhang besteht. Auf der Abbildung ist auch die errechnete Regressionsgleichung angegeben ; in der Gleichung ist — 2,682 die Richtungstangente, der von uns gesuchte Konzentrationsexponent, das andere Glied der Gleichung die konstante Zahl — 9,536. Dies wäre der Logarithmus der oben beschriebenen Konstante *a*. Auf der Ab-

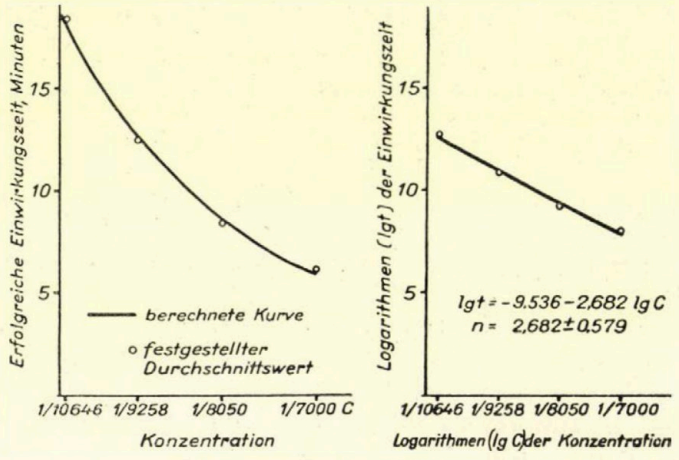

Abb. 1. Die erfolgreiche Einwirkungszeit der Desinfektion als Funktion der Neomagnol-Konzentration *(S. typhi)*

bildung wurden auch noch die Fehlergrenzen der Richtungstangente angeführt. Auf der linken Hälfte der Abbildung zeigen wir den Zusammenhang zwischen den Numeri der Konzentrationen und der Einwirkungszeiten. Hier gewinnen wir eine stark absteigende Kurvenlinie.

Auf Tabelle II wollen wir veranschaulichen, wie deutlich der von CHICK und WATSON festgestellte Zusammenhang auch aus unseren Beobachtungsresultaten entnommen werden kann. Am Fuss der Tabelle findet sich unsere auf Abb. 1 dargestellte Regressionsgleichung, hiervon gehen wir zu den Numeri über, nach deren Ordnung folgt die ursprüngliche Form der WATSONschen Gleichung mit dem Exponenten und der Konstante, die von uns errechnet wurden. In der dritten Kolonne der Tabelle zeigen wir die Produkte der 2,682-ten Potenz der Konzentrationen und der Einwirkungszeiten. Aus dem Vergleich der gewonnenen Zahlen geht hervor, dass das Produkt der Potenz der Konzentration und der Einwirkungszeit tatsächlich eine konstante Zahl ergibt. Die Schwankungen der einzelnen Werte liegen erheblich innerhalb der zulässigen statistischen Streuung.

Die praktische Bedeutung des dargestellten Zusammenhanges besteht darin, dass auf Grund seiner Werte die erfolgreiche Einwirkungszeit jeder

Tabelle II

Konzentrationsexponent von Neomagnol

Untersuchte Konzentration	Durchschnittliche erfolgreiche Einwirkungszeit in Min.	Produkt der Konzentrationspotenz und der Einwirkungszeit
C	t	$t\ C^{2,682}$
1 : 10646	18,7	$2{,}94 \cdot 10^{-10}$
1 : 9258	12,7	$2{,}90 \cdot 10^{-10}$
1 : 8050	8,5	$2{,}84 \cdot 10^{-10}$
1 : 7000	6,1	$2{,}97 \cdot 10^{-10}$
Errechnete Konstante		$2{,}91 \cdot 10^{-10}$

$$\log\ t = -9{,}536 - 2{,}682\ \lg C$$

$$t = 2{,}91.10^{-10} \cdot C^{-2{,}682}$$

$$tC^{2{,}682} = 2{,}91 \cdot 10^{-10}$$

Konzentration im voraus bestimmt und auch umgekehrt im voraus festgestellt werden kann, welche Konzentration jeder beliebigen Einwirkungszeit ent-

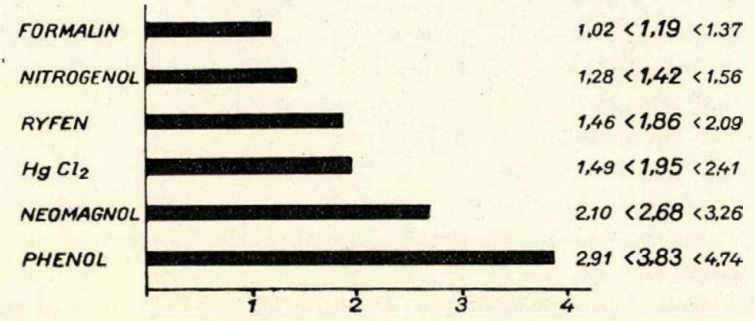

Abb. 2. n-Wert der Konzentrationsexponenten

spricht. Der Konzentrationsexponent stellt ein wichtiges Charakteristikum des Desinfektionsmittels dar. Bei Desinfizienzien, deren Konzentrationsexponent von 1 wenig abweicht, besteht zwischen der Konzentration und der erfolgreichen Einwirkungszeit einfach ein umgekehrtes Verhältnis. Wenn bei derartigen Desinfektionsmitteln die Konzentration z. B. auf die Hälfte herabgesetzt wird, steigt die Einwirkungszeit auf das Doppelte. Wesentlich anders verhalten sich jedoch Desinfektionsmittel mit verhältnismässig hohem Konzentrationsexponenten, wie z. B. Neomagnol oder Phenol. Bei diesen Desinfizienzien kann eine verhältnismässig geringe Veränderung der Konzentration

mit dem praktischen Aufhören der wirksamen Desinfektionswirkung einhergehen.

Auf Abb. 2 sind die Konzentrationsexponenten der von uns untersuchten 6 Desinfektionsmittel dargestellt. Am niedrigsten erwies sich der Konzentrationsexponent des Formalins. Bei diesem Desinfektionsmittel wird die Wirkung durch eine Konzentrationsveränderung am wenigsten beeinflusst. Ein verhältnismässig niedriger Konzentrationsexponent ist auch für Nitrogenol bezeichnend, wodurch die Vorteile dieser ungarischen quaternären Ammoniumverbindung hervorgehoben wurden. Ein ausgesprochen hoher Konzentrationsexponent und eine nach der Konzentrationsveränderung auftretende hochgradige Wirkungsverschiebung ist für Neomagnol und Phenol charakteristisch. Diese Feststellung in bezug auf Phenol stimmt mit den Literaturangaben überein.

Zusammenfassung

Zur Feststellung des Zusammenhanges zwischen den Konzentrationsveränderungen und den erfolgreichen Einwirkungszeiten wurden mit einigen alten und neuen Desinfektionsmitteln bei gegebener Temperatur und gegebener *Salmonella typhi*-Keimzahl Untersuchungen vorgenommen.

1. Ein anderes Bakterium verwendend als CHICK, konnte der von CHICK und WATSON beschriebene Zusammenhang bestätigt werden. Danach steht die Einwirkungszeit mit der je Desinfiziens variierenden *n*-ten Potenz der Konzentration im umgekehrten Verhältnis.

2. Unter den untersuchten Desinfektionsmitteln wurde ein verhältnismässig niedriger, d. h. 1 nahestehender Konzentrationsexponent bei Formalin und Nitrogenol festgestellt; als hoch erwies sich der Konzentrationsexponent von Neomagnol und Phenol.

3. Der Konzentrationsexponent ist ein wichtiges Charakteristikum der Desinfizienzien; ein hoher Konzentrationsexponent bedeutet, dass eine verhältnismässig geringe Veränderung der Konzentration mit dem Aufhören der Wirksamkeit der Desinfektionswirkung einhergehen kann.

4. Bei der Nachprüfung der Desinfizienzien im Laboratorium sollte in Zukunft auch die Bestimmung ihres Konzentrationsexponenten berücksichtigt werden.

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ЗАВИСИМОСТИ МЕЖДУ КОНЦЕНТРАЦИЕЙ И ДЕЙСТВИЕМ ДЕЗИНФИЦИРУЮЩИХ СРЕДСТВ

Й. СИТА и ДЬ. БАРШИ

Резюме

Авторы проводили эксперименты с различными прежними и новыми дезинфицирующими средствами при данной температуре и данному числу микробов Salmonella typhi с целью определения зависимости между концентрацией и временем успешного воздействия.

1. Авторы применяя иные бактерии, чем Чик, могли подтвердить описанную Чиком и Ватсоном зависимость. Согласно этому время воздействия находится в обратной зависимости со степенью -n концентраций, изменяющейся по дезинфицирующим средствам.

2. Из исследуемых дезинфицирующих средств сравнительно низкий, т. е. близкий к 1, концентрационный показатель, авторы нашли у формалина и нитрогенола, высокими оказались концентрационные показатели хлорогена и фенола.

3. Концентрационный экспонент особенно характерен для дезинфицирующих веществ, высокий концентрационный показатель означает, что сравнительно малое изменение концентрации может прекратить успех дезифицирующего действия.

4. Лабораторные контроли дезинфицирующих средств в будущем нужно распространить и на определение концентрационных показателей.

STUDIES ON THE OCCURRENCE OF POLIOMYELITIS VIRUSES IN HUNGARY

By

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The method introduced by ENDERS et al. [1, 2, 3] for the cultivation of poliomyelitis virus has made it possible to perform a more detailed study on the occurrence of this virus in Hungary, in order to obtain more accurate data concerning its rôle in this country. The investigation consisted in isolating and typing of strains and demonstrating the presence of specific antibodies.

Methods

Our method of tissue cultivation was essentially similar to that of ROBBINS et al. [3]. Skin and muscle of embryos from the 2nd to 4th month of pregnancy were cultivated in a thin layer of plasma at 36° C, in stationary cultures. The nutrient fluid consisted of 10 per cent chicken embryo extract, 5 per cent horse serum and 85 per cent HANKS' balanced salt solution, containing streptomycin and penicillin 50 μg each per ml.

Before inoculation of the cultures the nutrient fluid was removed and replaced by amounts of 0,2 ml of the inoculum. After about 30 minutes of contact, 1,8 ml of nutrient fluid was added and the cultures were put back into the incubator. The cultures were usually inoculated after 3 to 4 days of preliminary incubation. The fluid was changed every 4 or 5 days if observation was prolonged, or if indicated by the lowering of the pH. The nutrient fluid used for the second and further changes contained only 2,5 per cent horse serum and 5 per cent embryonic extract.

Isolation of the virus was attempted in most instances from faecal and occasionally from spinal cord samples. The 10 per cent suspensions of faeces were clarified by centrifugation first at 3000 then at 8000 r. p. m. To the supernatants thus obtained penicillin and streptomycin were added to yield concentrations of 1 mg and 2 mg per ml, respectively. From the spinal cord samples a 10 per cent suspension was made, and after a single centrifugation the supernatant was used for inoculation.

Neutralisation tests were carried out with a 1 : 10 final dilution of immune serum against different dilutions of the virus. After standing at room temperature for an hour, 0,2 ml of each serum-virus mixture were inoculated into tissue cultures. Sera neutralising 100 or more tissue culture LD_{50} of virus were regarded as positive.

In virus isolation experiments the cultures were observed for 8 to 10 days. From cultures not presenting any cytopathogenic lesions, subpassages were performed with pooled supernatants harvested on the 4th and 8th days. In titration and neutralisation experiments the final results were read on the 6th day.

Immune sera for typing purposes were prepared in rodents. Type I and III poliomyelitis viruses were administered intravenously to rabbits, in the form of tissue culture fluid. Seven inoculations were made, each with 10 ml, at intervals of five days. The animals were bled on the 10th day following the last inoculation. The neutralisation indices of the immune sera thus obtained were higher than 1000. Antibodies against type II poliomyelitis viruses were produced in guinea pigs. As a vaccine, a suspension made from the brain and cord of mice infected with the Lansing strain was used, in doses of 1 ml, given intraperitoneally at three instances. The neutralisation indices of the sera obtained ranged from 100 to 1000.

Strains of virus

In immunisation experiments and neutralisation tests the Brunhilde and Leon strains of type I and III poliomyelitis viruses were used. The specificity of the immune sera was controlled later with the Mahoney and Saukett strains. For production of type II immune sera the Lansing strain was used. Control and neutralisation tests in this case were performed with the MEF1 strain. The strains Brunhilde, Mahoney, MEF1, Leon and Saukett were kindly supplied by the Virus Department of the State Institute of Hygiene (Budapest), in the form of tissue culture supernatants. The Lansing strain was obtained from DR. GALLIA (Prague).

Results

The isolation of poliomyelitis virus was attempted from a total of 54 samples. Of these, 48 were faeces from acute patients and 6 were spinal cords obtained from fatal cases. Samples were inoculated into 4 to 6 tissue culture tubes each and two successive passages were always made. Only the results of the third passage were evaluated. Isolation was attempted at least twice from each sample.

Eighteen faecal samples out of a total of 48, were found contaminated with bacteria in spite of the addition of streptomycin and penicillin. 22 bacteriologically sterile samples exerted a more or less marked toxic effect on tissue cultures. As from these toxic materials we succeeded in isolating 7 strains of poliomyelitis virus while from the 8 non-toxic ones 2 strains, it seemed that there was no difference in the frequency of successful isolations according to the toxic or non-toxic properties of the samples. 2 of the 6 spinal cord samples were found to be contaminated with bacteria, and from the remaining 4 samples 1 strain was isolated.

Table I

Neutralisation test for typing the cytopathogenic agents isolated from patients with poliomyelitis

Strains isolated	Immune sera		
	Type I	Type II	Type III
F 0	—	—	pos.
G 15	—	pos.	—
F 30	pos.	—	—
F 32	pos.	—	—
F 36	pos.	—	—
F 38	pos.	—	—
F 39	—	pos.	—
F 46	—	pos.	—
F 49	—	pos.	—
F 50	—	pos.	—

pos. : Neutralisation test positive
— : Neutralisation test negative

The cytopathogenic agents thus isolated were serially transferred and from time to time titrated. In spite of the passages, the titre of some strains never exceeded 10^{-2}, while that of the others reached 10^{-5}. Isolated strains were immediately inoculated intracerebrally into white mice, but no symptoms were observed. Suckling mice proved equally insusceptible to these viruses.

It has been attempted to identify the strains by means of the immune sera available. The results of repeated neutralisation tests, summarised in Table I, clearly show that all the strains isolated were poliomyelitis viruses. Out of the 10 strains isolated 4 belonged to type I, 5 to type II, and 1 to type III.

Parallel with the virus isolation experiments, studies concerning the frequency of antibodies against the different types of poliomyelitis viruses in healthy persons were also carried out. The results of the neutralisation tests carried out with the three types of poliomyelitis viruses and the sera of persons of different age groups are summarised in Table II.

Table II

The frequency of occurrence of neutralising antibodies against the three types of poliomyelitis virus in healthy persons

Age group	Number of subjets	Type I		Type II		Type III	
		positive	per cent	positive	per cent	positive	per cent
1/2—2 years	21	6	28	2	9	4	19
3—7 years	29	17	59	13	45	16	55
8—15 years	39	23	59	29	74	21	54
16—30 years	37	27	73	30	81	25	67

It can be seen from Table II that the frequency of occurrence of antibodies increases with age. A parallelism in the occurrence of type I and III antibodies could be observed while that of type II exhibited a somewhat different pattern.

Discussion

The investigation was carried out in order to attempt isolation of poliomyelitis virus in human embryonic tissue cultures. Ten strains could be isolated, namely 9 from faeces and 1 from a spinal cord. Only from one third of the adequate faecal samples was the isolation successful. This ratio is especially low, as most of the samples have been obtained from typical cases of poliomyelitis. As to the cause of the low percentage, transport of the samples, often exceeding the time desirable, might have caused a lowering of the virus content. However, the sensitivity of the method employed is not the highest

either, as, according to some more recent data, the probability of isolation is higher in monolayer epithelial than in the usual fibroblast cultures [4].

Before the present studies, only the isolation of type I poliomyelitis virus had been reported in Hungary [5]. It was remarkable that, out of the 10 strains isolated during the epidemic in 1954, 5 belonged to type II. Since 4 strains of type II were isolated from patients admitted at the same time to the Department of Paediatrics at Szeged, there was probably a local accumulation of type II infections. A similar observation has been made by ROBBINS et al. [6] who, out of 13 strains isolated, identified 10 strains as belonging to type III.

From the results of the neutralisation tests, it could be established that the conditions of infection in Hungarian towns are similar to those prevailing in other civilized countries. Our studies carried out with the sera of healthy inhabitants of Szeged have shown that the appearance of antibodies against type I and III poliomyelitis viruses exhibits an intimate parallelism. Characteristically the occurrence of antibody does not change in the age groups between 3 and 15 years, since it attains the 50 per cent value before the third year. It seems, therefore, that most of the infections with these two types are contracted in early childhood. The frequency of type II antibodies in the sera tested was more regularly related to the age than that of the other two types, and was in good agreement with our earlier observations made in 1951 by Lansing virus neutralisation tests in mice [7].

Summary

1. We have succeeded in isolating in 1954 10 poliomyelitis virus strains in Hungary from patients with poliomyelitis, by making use of human embryonic tissue cultures. Nine of the strains have been isolated from faeces and one from a spinal cord. Typing was made by immune sera, produced in the guinea pig for type II and in rabbits for types I and III. Four strains were found to belong to type I, five to type II and one to type III.

2. Neutralising antibodies against all the three types have been demonstrated at a varying ratio in the sera of healthy persons. The frequency of occurrence of the antibodies rose with age. In the adult population ; 73 per cent were type I, 81 per cent type II, and 67 per cent were type III strains.

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ИЗУЧЕНИЕ ВСТРЕЧАЕМОСТИ ВИРУСОВ ПОЛИОМИЭЛИТА В ВЕНГРИИ

М. ПИНТЕР, Э. АБРАХАМ и М. РАВНАИ

Резюме

1. От больных полиомиэлитом в Венгрии в 1954 году удалось изолировать десять штаммов вируса полиомиэлита в культурах из человеческой эмбриональной ткани. Материалом исследования в девяти случаях служили испражнения, а в одном случае — спинной мозг. Штаммы могли быть типизированы в тканевых культурах с помощью иммунных сывороток, полученных от морских свинок (тип 2.) и от кроликов (тип 1. и 3.). К типу 1 относятся четыре штамма, к типу 2 — пять штаммов и к типу 3 — один штамм.

2. В сыворотках здоровых лиц в различных отношениях обнаруживались вирус-нейтрализующие антитела ко всем трём типам штаммов. Встречаемость антител повышается с возрастом. У взрослых авторы определили следующие отношения встречаемости: тип 1: 73%, тип 2: 81%, тип 3: 67%.

GROWTH OF MM VIRUS IN HUMAN EMBRYONIC TISSUE

By

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Since the virus strains belonging to the encephalomyocarditis (EMC) group, such as the Col SK, MM, EMC, Mengo, strains had been discovered, the biological properties of this virus group have been dealt with in numerous reports. Its relation to poliomyelitis virus was a particularly controversial issue, so long as it has not been established that no serological relationship existed between the two groups of virus [1, 2]. All its strains having been isolated from animals, there was doubt about the pathogenicity of EMC virus for man. At first, only serological evidence had suggested that they might cause disease in man [3]; more recently, however, the virus was isolated also from human cases [4, 5, 6]. According to present knowledge, in grave cases there are encephalomyelitis and/or serous meningitis, but abortive and asymptomatic forms may also occur.

In general, the viruses of the EMC group grow readily in the chicken embryo and tissue cultures alike. The pertaining reports deal mainly with the cultivation of the Col SK and MM viruses, but it can be assumed that the other members of the group do not greatly differ in cultural properties. SANDERS and JUNGEBLUT [7] studied the growth of MM virus in different Maitland-type cultures of chicken embryo and mouse embryo tissues and found the virus passable in cultures prepared from embryonic chicken tissue, embryonic mouse brain and lung. CHAMBERS et al. [8] showed that the MM virus grew slowly and slightly in suspended mouse and human testicle tissue cultures but could not systematically passage the virus. More recently, SMITH and EVANS [9] have used monkey testicle, and FABIYI [10] mouse testicle roller tube cultures for cultivating MM virus and found that the virus grew abundantly in these cultures. The multiplication of virus in both tissues is accompanied by a degeneration of fibroblasts.

In the following, we shall report on the growth and cell pathogenicity of MM virus cultured in human embryonic tissue.

Materials and methods

The strain of MM virus used in the experiments has been obtained from DR. W. RHODE (Jena), in the form of infected mouse brain. The strain was passaged and found to have a mouse infectivity titre varying from 10^{-6} to 10^{-8}. The animals infected died in 2 to 5 days, with the characteristic symptoms of encephalomyelitis.

Multiplication of virus was studied in cultures prepared from skin and muscle from 2 to 4-month-old human embryos. The pieces of tissue were cultured at 36 °C, in a thin layer of hen plasma, with the stationary tube culture technique. The nutrient fluid, of which 2 ml was added to each tube, consisted of chicken embryo extract 10 per cent ; horse serum 5 per cent ; HANKS' solution, 85 per cent ; with 50 μg of penicillin and 50 μg of streptomycin per ml. Infection was carried out with 0,2 ml of inoculum on the 3rd or 4th day of culturing. Before adding the new nutrient fluid, the tissues were allowed to remain in contact with the inoculated material for 30 minutes.

Tenfold-diluted viral material and serum of 1 : 10 end dilution were used in the neutralisation tests. The serum-virus mixture was kept at room temperature for 1 hour and after that 0,2 ml of it were transferred into each tube.

The specific antiserum to MM virus was produced in rabbits. Each rabbit was inoculated three times in intervals of one week, with 1,0 ml of a 10 per cent suspension of MM virus-infected mouse brain, by the intramuscular route. 10 days following the last inoculation the animals were bled. The sera obtained had a neutralisation index of 10 000, as determined in the mouse.

Results

The starting material was a mouse brain suspension of $10^{-6.5}$ titre, tenfold dilutions of which were used for infecting the human embryonic tissue cultures. A 10^{-1} dilution of this viral material 18 hours after infection produced a clearly visible degeneration of fibroblasts and after 48 hours no normal cells were visible in the culture. At a dilution of 10^{-7} the degeneration developed on the third day, while a 10^{-8} dilution produced no cell lesion. The degenerating cells were characteristically elongated and contained fine granules in the cytoplasm. Another conspicuous feature was that, even with advanced degeneration, the cells remained grouped in their original, radial order. These characteristics make it possible to distinguish to a certain extent the cytopathogenic changes produced by the virus from those brought about by the poliomyelitis virus.

Next, by passaging the virus in tissue cultures the 16th passage was reached. During passage we titrated the virus in tissue culture on 5 occasions and found the titre to vary between 10^{-4} and 10^{-6}. In two cases parallel titrations were carried out in the mouse ; the titres yielded were in satisfactory agreement with those obtained in the tissue culture. Fluid from the 7th passage was examined by the neutralisation test, in an effort to determine whether the cytopathogenic agent passaged in the tissue culture was identical with the original MM virus. The rabbit sera, which had proved specific in mouse experiments, neutralised up to 10 000 tissue culture LD_{50}. In view of this the virus passaged in the mouse may be considered identical with that passaged in tissue culture.

In the 9th passage the growth curve of the virus was established. Each of a number of tubes was infected with 1000 LD_{50} of virus and after 30 minutes the tubes were washed with Hanks' solution to remove virus not bound to cells.

Nutrient fluid was then added and cultivation was continued in the usual manner. Meanwhile, at regular intervals the contents of 4 tubes were pooled and the fluids thus collected were stored at —20 °C. The pools were then titrated in tissue culture.

In Fig. 1 are shown the titres obtained at different points of time. After 6 hours the titre was as low as 10^{-1}, meaning that only part of the inoculated virus could be demonstrated. The rate of multiplication then increased rapidly, to reach the maximum titre of 10^{-5} in the 24th hour. Subsequently the titre

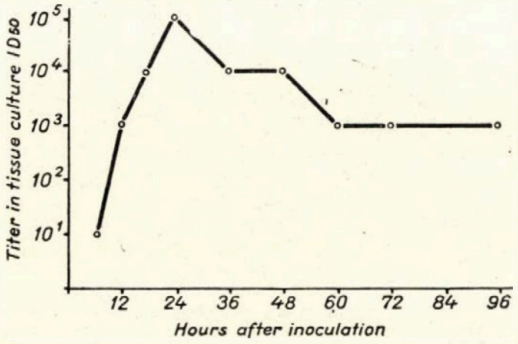

Fig. 1. Growth of MM virus in human embryonic tissue culture

slowly declined, but as late as after 96 hours a demonstrable quantity of virus was still considerable. As to the relation between multiplication rate and development of cytopathogenic action, the latter was found somewhat to lag behind the former, inasmuch as the complete degeneration of cells took as much as 48 to 64 hours.

Discussion

We have succeeded in growing MM virus, a member of the EMC group, in human embryonic tissue. In the only report on successful growth of MM virus in human tissue [8] no clear-cut evidence can be found as regards the relationship of the virus to human tissue. Our results indicate that in human embryonic tissue the MM virus multiplies at the same rate as in mouse or monkey tissue cultures. This agrees with the clinical observations, which all suggest that the MM virus is capable of growing in the human organism. VERLINDE and VAN TONGEREN [6] have contributed valuable evidence to this point when they recovered a virus strain belonging to the EMC-group from a case believed to be one of poliomyelitis, and from another showing the symptoms of encephalomyelitis.

Our method for growing the MM virus seems to be suitable for use in neutralisation tests on a larger scale. The method is less costly than the mouse

test and is apparently more specific than the haemagglutination-inhibition test. In informative trials attempts have been made to use the method for the demonstration of antibody. Sera from 165 normal individuals were examined by the neutralisation test and the presence of neutralising antibodies could be shown even by repeated testing in 4 cases. These investigations still await corroboration by animal experiments and will therefore not be evaluated here. Should the positive sera possess a specific neutralising action, this will be interpreted as a proof of the occurrence of viruses belonging to the EMC group virus in Hungary.

Summary

The MM virus can be grown readily in fibroblast culture prepared from human embryonic skin. The virus reaches its maximum titre (10^{-5}) in 24 hours and causes meanwhile an initial degeneration of cells, resulting ultimately in total tissue destruction. The method described is suitable also for use in neutralisation tests.

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КУЛЬТИВИРОВАНИЕ ВИРУСА ММ В ЧЕЛОВЕЧЕСКИХ ЭМБРИОНАЛЬНЫХ ТКАНЯХ

М. ПИНТЕР и Э. АБРАХАМ

Р е з ю м е

Вирус ММ, относящийся к группе энцефаломиокардитов, хорошо размножается в культуре фибробластов из кожи и мускулатуры человеческого зародыша. В 24-ом часу после инокуляции титр вируса достигает максимального значения (10^{-5}). Одновременно с размножением вируса наблюдается дегенерация клеток, и процесс этот приводит к полной гибели клеток. Описанный способ культивирования пригоден для постановки реакций нейтрализации. С помощью реакции нейтрализации в сыворотках нескольких процентов здоровых лиц удалось обнаружить антитела, нейтрализующие вирус ММ.

BACTERIAL SOFT ROT IN GREEN PEPPER (CAPSICUM ANNUUM)

By

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(Received, January 24, 1956)

During 1954 and 1955, from various parts of the country samples of green pepper (paprika) were being sent to our Institute, bearing symptoms of a soft, slimy decay on the fruits, but no lesions at all in the foliage. Incidences were being reported by most of the paprika growers in the country. They have known the disease for long, but the pathogenic agent was unknown to them.

Symptoms

Initially the disease attacks only the fruit, in its various stages of ripening, from pollination to full ripeness. On the young green fruit a soft rotting process, from light brown to olive brown in colour, is seen to extend rapidly. Usually, and in some varieties almost exclusively, the part next to the calyx is attacked and from there the infection spreads to the entire tissue in dependence on the obtaining environmental conditions. The epicarp, however, remains unaffected, so that the disintegrating parenchyma cells accumulate as a malodorous fluid in the tip of the fruit (Fig. 4). With the weather turning dry, the slimy liquid inside the pod slowly dries up and the epicarp hardens parchmentlike (Fig. 2b). If, however, the weather remains continuously moist, the rotting process gradually spreads to the capsule, and via the capsule to the calyx, to reach later the peduncle. In some instances rotting is so very swift that the rotted tissues become detached from the calyx, and the disintegrated fruit falls to the ground (Fig. 2a). When still young, the diseased fruit is frequently thrown off together with the peduncle (Fig. 3). In this manner the infection is prevented from reaching the stalk because, if the stalk is affected, although this occurs seldom and only superficially, its parts above the infected spot will shed their leaves and die off. In affected peduncles and stalks the rotting process is slower than in the fruit and manifests itself in spots which are first dark olive green, to turn brown while drying.

In fruits in the ripening stage or already perfectly red, the process is the same as in young green fruits, except that there occurs no change in the colour.

Disintegration of the tissue even when freshly infected, was so very rapid that it was impossible to prepare sections for the study of their pathological changes. Under the miscroscope, the disintegrating cells were found to contain innumerable exceedingly motile bacteria and to yield an almost pure culture of the pathogenic agent. When cutting an infected pod, at the areas corresponding to the brown infected spots, the endocarp was seen to be covered by a slimy, yellowish brown coating consisting of bacterial masses.

Isolation and identification of the pathogenic agent

Isolations were performed on bouillon agar. The colonies developed rather rapidly ; in 48 to 72 hours they turned yellow, became circular, smooth, glistening, and displayed entire margins ; initially, the latter showed an opalescent white. It was rather striking that on artificial media, in the course of repeated subculturing, the colonies gradually lost their yellow colour and turned a creamy opalescent white, imparting at the same time a slightly green fluorescence to those media. At first, this phenomenon intrigued us into believing that our culture had become contaminated, but our fears were dispelled on seeing that the pathogenicity and the morphological and physical properties had remained the same throughout. Rods measuring 0,5 μ by 1,3 to 1,7 μ were found, with rounded ends, very motile, not forming spores, Gram negative, somewhat liquefying gelatine, forming ammonia in peptone water, not forming indole and hydrogen sulphide, slightly reducing nitrate to nitrite. In litmus milk they formed a slight curd at the bottom of the tube after 4 days ; the liquid above it was slightly peptonized and the litmus reduced. There was some hydrolysis of soluble starch on beef extract agar ; acid was produced in 22 days, markedly in glucose, galactose, arabinose, mannose ; less markedly in saccharose, maltose, mannitol, and glycerin. No acid was produced from lactose, raffinose, dextrin, dulcite, and asparagine.

On the evidence of these morphological, physiological, and biochemical data, this organism is similar to *Pseudomonas syringae* van Hall [2], although it differs from it in some respects, primarily in the colour and the type of the colony.

Soft rot in the green pepper fruit was first described by ORSINI in 1942 [4], in the autumn of which year it was widespread in Perugia, Central Italy. The symptoms described by him are exactly the same as those of the bacterial soft rot observed in our country. The disease has so far not been reported from any other country. ORSINI has termed the organism *Bacterium syringae var. capsici* Orsini. Unfortunately, instead of giving a morphological and physiological description of the organism isolated from green pepper, he only mentions in his paper that in its morphological and physiological characteristics the organ-

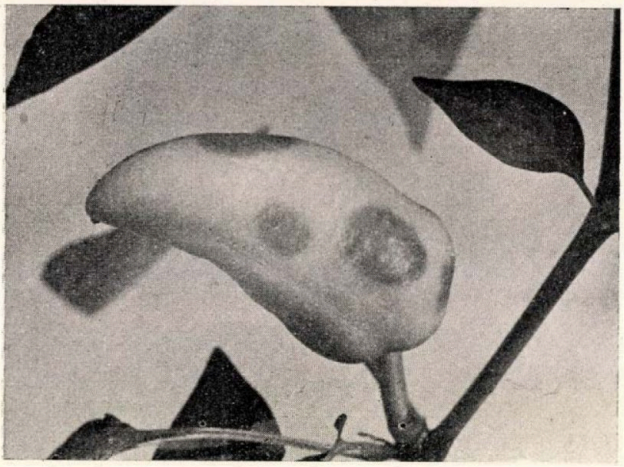

Fig. 1. Green pepper fruit 3 days after inoculation

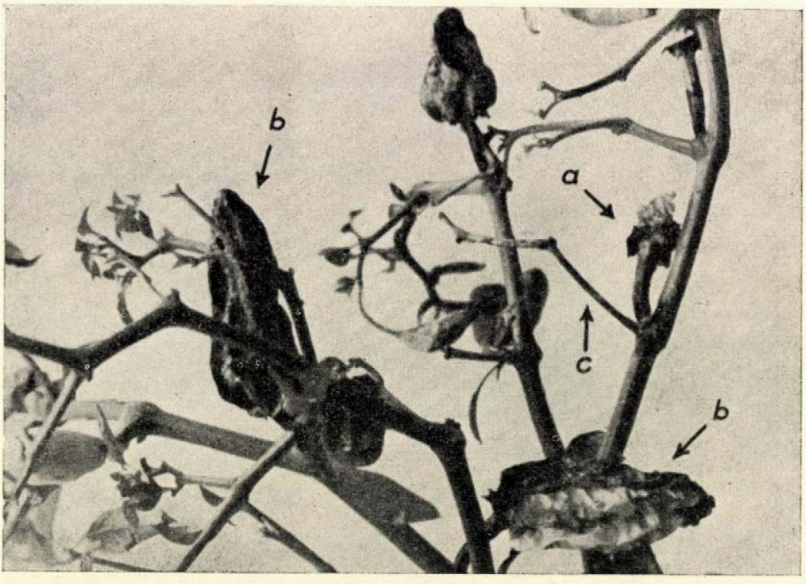

Fig. 2. Green pepper plant severely infected under natural conditions : *a)* peduncles still attaching ; *b)* fruits mummified parchmentlike ; *c)* necrosed portions of branches

Fig. 3. Green pepper on the 6th day after inoculation in the field. The younger fruits fell to the ground, peduncles included

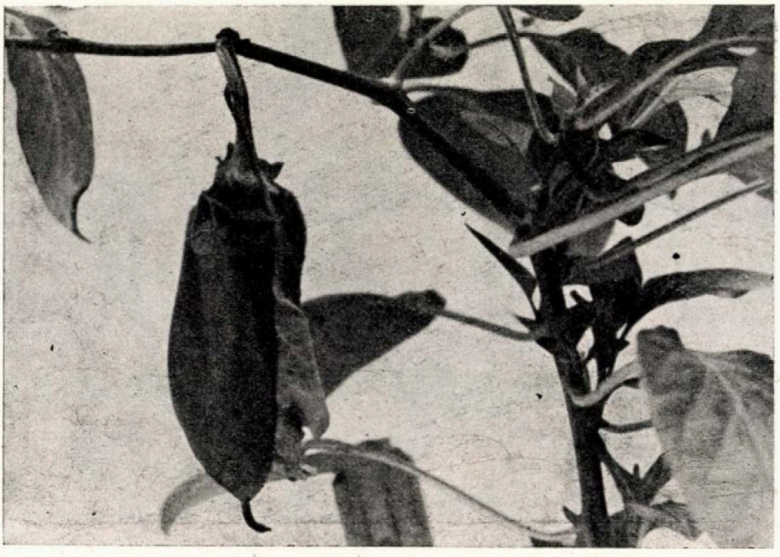

Fig. 4. Tissue fragments within the epicarp accumulated in the tip of the fruit

ism has been found to be very similar to *Bacterium syringae* (van Hall) E. F. Smith. However, as ORSINI had failed to infect with the new microorganism the green shoots of lemon, lilac, and pear, to all of which *Bacterium (Pseudomonas) syringae* is pathogenic, he finally arrived at the conclusion that the organism causing soft rot in green pepper is to be regarded as a biological race of *Bacterium syringae* which has specialised and adapted itself to green pepper. It was on this account that he designated the agent as *Bacterium syringae* (van Hall) E. F. Smith *var. capsici* Orsini.

Since in respect of pathogenicity the organism detected in Hungary is exactly the same as the one described by ORSINI, and as its morphological and physiological characteristics are very similar to those of *Bacterium (Pseudomonas) syringae* (van Hall) E. F. Smith, we regard it as identical with *Bacterium syringae* (van Hall) E. F. Smith *var. capsici* Orsini.

However, on the grounds that the pathogenic agent responsible for soft rot in pepper imparts to the nutrient medium a green fluorescence, and on account of a number of its other properties, we assign to it in accordance with the rules of the new nomenclature the designation *Pseudomonas syringae* van Hall *var. capsici* (Orsini) Klement.

On the question whether the organism which is the causative agent of soft rot in pepper can at all be regarded as a biological race of *Pseudomonas syringae* one should keep an open mind until further investigations have been made. ORSINI himself has pointed out [4] the necessity of an exact and full identification of the two microorganisms. It would appear that before a firm stand can be taken concerning the ultimate place for this new organism "within the *Pseudomonas syringae* group", to which microorganisms described by many authors are referred, the entire group must be further studied serologically and in comparative experiments.

There are other bacterial agents equally capable of inducing rot in green pepper [1, 5, 6], but attempts to isolate these organisms have so far failed.

Infection experiments

Infection experiments were carried out to obtain confirmation of the pathogenicity of the organism. Green pepper fruits were inoculated with a sterile aqueous suspension of the pure culture, by pricking them with a needle fixed between the hairs of a brush. Under greenhouse conditions, at 20 to 25 C°, the infection was as soon as in 24 hours seen to cause an oozy brown spot around the wound. Keeping the shape of a regular circle, this spot continued spreading and by the fifth or sixth day half the fruit was rotten (Fig. 1). By the eighth or tenth day, the mellow decomposed flesh of the entire fruit concentrated in the tip, and the fruit either remained attached to the peduncle or became

detached from the calyx through its own weight. Capsule, calyx, border, and peduncle were likewise rotten, but the epicarp remained unaffected.

Under field conditions, plots containing 100 plants of green pepper of the Cecei variety each were infected, in part by pricking, in part by spraying them, at the end of August and the beginning of September. In the fruits infected by wounding, the process of rotting run as rapid a course in the field as in the greenhouse (Fig. 3), despite the fact that there was no rainfall in the days following inoculation. The young fruits together with their peduncles became detached in 3 to 4 days, while the larger, more developed ones remained on the plants. The infected fruits perished without exception.

Not more than 0,5 to 1 per cent of the fruits infected by spraying showed symptoms of soft rot, and this proves that the organism is unable to penetrate through the intact skin. No infection has been observed to arise unless there was a break, a discontinuance, somewhere between the skin and the calyx through which the bacterium could pervade the capsule.

No spots were caused on the leaves.

Like ORSINI, we have succeeded in artificially infecting tomato fruits by way of wounding them. In these, the rotting process was a slower one and the fruits became disintegrated in their entirety.

Conditions of incidence

In Italy the disease makes its appearance when after a few rainy days there is a drop in temperature ; it then spreads rapidly over whole plots. In Central Italy, this period is at the end of October and the beginning of November. In Hungary, too, severe outbreaks could often be observed in September and October, though sometimes they occurred in August as, for instance, in 1954 at Cegléd, during the coolish weather that followed the rains usual in that district in the first half of the month. The relatively dry summers do not favour the spread of the disease in Italy, while the sudden drops in temperature that follow thunderstorms in Hungary are conducive to it.

There is experimental evidence to prove that no infection takes place unless the epicarp is injured. Injuries can be due to various causes. They may be caused during some of the agronomical operations. Most frequently, and particularly on sandy soils, crystals of quartz wind-borne in sudden downpours inflict wounds upon the plants. Slight gaps between calyx and epicarp likewise furnish routes for penetration, and as a matter of fact constitute the most natural point of departure for soft rot. This particularly holds for varieties in which the pod is concave at this site. The infectious fluid pouring out and spreading from diseased fruits, or washed off them by the rain, is held up on the other fruit and stagnates at the place where calyx and epicarp meet, thereby inviting infection.

Fruits destroyed by soft rot furnish the infective material in large amounts. Due to biotic and abiotic causes, their walls burst, the infective contents pour down on the fruits below them, or pervade the soil.

Simultaneously, or even associated with the disease, Botrytis infections occasionally arise [3], or the disease may pave the way for different parasitic or saprophytic fungi.

Control of the disease

Since there are no effective chemical means of control known, other measures must be resorted to.

1. Affected fruits must be removed and destroyed at once, before the epicarp bursts and the infective fluid spreads. Growers frequently commit the mistake of leaving the affected fruits on the plants, since they have no market value.

2. The disease does not spread by the seeds, consequently it is always the rotted fruits and their infective contents pervading the soil, that are responsible for outbreaks in the next year. For this reason, rotation is of major importance, and green pepper must not be followed by green pepper.

Summary

After Italy, *Bacterium syringae* (van Hall) E. F. Smith *var. capsici* Orsini, the bacterium responsible for soft rot in green pepper fruits, has been described from Hungary, where it spread all over the country in the cool periods following thunderstorms, and in September and October. The organism attacks the fruits but not the leaves. Exceptionally, the disease extends to the peduncle and from there to the stalk. Both greenhouse and field infections provided evidence for the pathogenicity of the bacterium. Under experimental conditions it was possible to infect tomato fruits as well.

Because it imparts a fluorescent green colour to the nutrient medium, and on account of several of its other properties, according to the international rules of nomenclature the agent has been listed with the genus *Pseudomonas*, and the designation *Pseudomonas syringae* van Hall *var. capsici* (Orsini) Klement has been suggested for it.

Whether this pathogenic agent can be regarded as a biological race of *Pseudomonas syringae* is a question which should be left open until further investigations will have been made.

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БАКТЕРИАЛЬНОЕ МЯГКОЕ ГНИЕНИЕ СТРУЧКОВОГО ПЕРЦА

З. КЛЕМЕНТ

Резюме

После Италии впервые в нашей стране была обнаружена бактерия, вызывающая мягкое гниение плодов стручкового перца, Bacterium syringae (van Hall) E. F. Smit var. capsici Orsini. Болезнь распространилась повсеместно после охлаждения погоды, наступающего вслед за грозами, а также в сентябре и октябре. Возбудитель болезни поражает только плод и не повреждает листвы. В некоторых случаях болезнь через ножку может перейти и на стебель. Путем экспериментов, произведенных в парниках и на открытом воздухе, автор доказал патогенность бактерии. При искусственных условиях удалось заразить также и плоды помидоров.

Так как возбудитель, по производству им зеленой флуоресцентной краски в питательной среде и по другим особенностям, — согласно новых правил номенклатуры — причисляется к роду Pseudomonas, то предлагаем для возбудителя мягкого гниения стручкового перца дать название Pseudomonas syringae van Hall var. capsici (Osrini) Klement.

Рекомендуется, однако, в дальнейшем исследовать, можно ли данный возбудитель считать биологической расой Pseudomonas syringae.

SEROLOGICAL TYPING OF PROTEUS STRAINS FROM INFANTILE ENTERITIS AND OTHER SOURCES

By

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The potential pathogenicity of Proteus and related organisms has been known since the earliest days of bacteriology. Although these bacteria are widely distributed in nature, they are able under certain conditions to give rise to pathological disturbances in the human body. The role they play in infantile enteritis is not yet entirely clear, but there is no doubt that they are primarily responsible for some of the cases.

The frequent occurrence of these bacteria both in pathological and non-pathological materials indicates the importance of the differentiation among various strains, since in this way further knowledge may be gained of the supposed pathogenic role of certain cultural or serological types.

Proteus and related organisms are divided into groups on the basis of their biochemical behaviour. For further differentiation among strains of these groups the serological method is the only practical means. The antigenic structure of these bacteria has been studied by a number of workers. *P. morganii* strains were isolated by RAUSS in 1936 from cases of enteritis and classified by O and H antigens [1]. Numerous antigenic types have been recognized within the *P. rettgeri* group and within the *Providencia* group. The serology of the largest and most frequently occurring group, *P. hauseri*, was investigated by WINKLE in 1944 [2]. The more detailed and extensive studies of KAUFFMANN and PERCH have served to establish a satisfactory serological basis for the identification of these organisms. [3] Later, PERCH completed this antigenic schema with regard to the partial antigens, showing 49 O groups and 110 types [4].

The present work has been undertaken to gain information as to the biochemical behaviour and antigenic structure of Proteus strains from faecal samples, and also to study the frequency of the different types of these bacteria in normal and pathological material. The serological examination of *P. hauseri* strains was based on the KAUFFMANN—PERCH simplified antigenic schema. A new technique had to be employed for O agglutination. According to PERCH, determination of the somatic antigen of Proteus strains should be done with boiled cultures by tube agglutinations as living cultures are often inagglutinable in O sera. This technique, however, makes the routine diagnosis very difficult.

Accordingly, one of the chief objects of this work has been to work out a method which makes the exact O antigen determination possible by slide agglutination.

Materials and methods

1. Isolation of cultures

Faecal specimens from cases of infantile enteric infections were sent to this laboratory for routine examination by various hospitals and physicians. All the patients of this group were under one year of age. The samples were collected without taking into consideration whether or not the patients had been receiving antibiotics. Control specimens from normal infants also under one year of age, were collected from several nurseries. Stools from suspected cases of dysentery were obtained from our routine material. Stools from food handlers served as material from normal adults.

Proteus strains from faecal samples were isolated on ENDO's, desoxycholate citrate (D.C.) (SERÉNY, [5]) or bismuth sulfite (Bi) agar (LOVREKOVICH, [6]), and these media were also used for the detection of Salmonella, Shigella and pathogenic *E. coli* strains. The faeces were streaked directly on plates of these media. The two selective media were heavily inoculated, while only a smaller amount was plated on Endo's agar. No fluid enrichment media were used. Inoculated media were read after incubation for 20 to 24 hours at 37° C, then left to stand for another 24 hours at room temperature and read again. From the plates only one or a few colonies were investigated. Proteus strains occurring in mixed culture were separated on D. C. and/or Bi agar.

2. Biochemical reactions

All the isolated strains were subjected to the following biochemical reactions.

Indole reaction : peptone water incubated for two days and tested with Ehrlich's reagent.

Urease test : Szita's fluid medium [7].

Formation of H_2S : Lead acetate agar.

Fermentation of sucrose, maltose, mannitol and xylose : 1 per cent in peptone water, Andrade's indicator. Observed for 14 days. Only *P. morganii* strains were tested in liquid *ammonium sodium citrate* medium ; observation lasted for 4 days.

3. Preparation of sera

H and O sera necessary in the type diagnosis of *P. hauseri* were prepared from test strains of the KAUFFMANN—PERCH schema. For the preparation of H antigens the growth of swarm agar slants was suspended, each in 6 to 8 ml of formalin-saline.

O antigens were obtained by suspending the growth of agar plates in saline and steaming the suspension without pressure for 2½ hours. The boiled bacteria were washed twice in saline, then resuspended and used for immunization. As shown by PERCH, simple boiling of Proteus cultures will not give an antigen which would produce pure O agglutinins, especially not with a certain variation of strains ("dull form"). For slide agglutination, O sera free from H agglutinins being essential, the washing of suspensions is always necessary.

Rabbits were injected at intervals of 4 days with subsequent doses of 0,5, 1,0, 2,0, 4,0 ml of an H antigen suspension or 0,1, 1,0, 2,0, 4,0, 5,0 ml of an O antigen suspension, respectively. Both O and H antigen suspensions contained approximately $2 \cdot 10^9$ organisms per ml.

Determination of antigens and control examination of serological technique

1. Determination of H antigens

For flagellar agglutination, slide agglutination with living bacteria from soft agar slants was performed. The slants had been inoculated in the water of condensation on the previous day. H sera at dilutions of 1 : 100 to 1 : 500

gave prompt flagellar agglutination and, as a result of these high dilutions, the effect of O agglutinins rarely disturbed the diagnosis. Typing becomes more exact, however, by using absorbed sera or H sera obtained from strains of an O group different from the strain under investigation. Five pooled H sera were prepared and used at dilutions of 1 : 20 to 1 : 50 :

H : 1—19
H : 1, 2, 3
H : 4, 5, 6, 7, 8
H : 10, 11, 12, 13, 14
H : 15, 16, 17, 18, 19

Strains isolated on selective media, especially on D. C. agar, often bore poorly developed flagella. For the H antigen determination of these cultures a passage was necessary through semisolid agar.

2. Determination of O antigens

The O inagglutinability of living Proteus cultures can be eliminated by using desoxycholate citrate (D. C.) agar. The flagellar antigen of strains growing on this medium is so underdeveloped that the cultures are often inagglutinable in H sera. The growth of several Proteus strains on D. C. agar is often poor ; if the medium is heavily inoculated, however, this inhibition is usually not apparent.

For slide agglutination with living cultures from D. C. agar, the O sera could be diluted from 1 : 20 to 1 : 50. Sera were so diluted that agglutinations due to minor, diagnostically not important antigens could be excluded. 13 pooled O polyvalent sera were prepared.

A : $A_1 + A_2$
A_1 : 2, 4, 5, 6, 9, 43
A_2 : 8, 21, 36, 45, 48
B : 15, 16, 17, 18, 20, 33, 35, 40, 41, 49
C : $C_1 + C_2$
C_1 : 1, 3, 26, 28
C_2 : 7, 10, 11, 12, 14, 47
D : $D_1 + D_2$
D_1 : 19, 22, 24, 25
D_2 : 27, 29, 32, 34
E : $E_1 + E_2$
E_1 : 13, 23, 30, 37, 38
E_2 : 31, 39, 42, 44, 46

In composing the polyvalent sera, minor antigenic relationships were also considered. Polyvalent O sera were used at dilutions of 1 : 5 or 1 : 10. O agglutination under these circumstances appeared as a prompt, granular aggregation of bacteria, sometimes, however, resembling to agglutination of the K type.

In typing the isolated cultures, slide agglutination tests with pooled O

sera were employed for preliminary examination, while the O group of the cultures was determined by examination in the separate O sera. Cultures that gave strong reactions in one of the O sera were regarded as belonging to the same O group. It is obvious that the O antigens of these cultures were not necessarily identical with the O antigen of the strain used for preparing the corresponding O serum. This is naturally also true regarding the flagellar antigens.

3. Examination of the Kauffmann—Perch schema by the new agglutination method

By the slide agglutination technique described above, test strains received from the State Serum Institute, Copenhagen, were examined against all O and H sera prepared from these strains. Our results completely corresponded to the KAUFFMANN—PERCH schema.

Cross agglutinations due to minor O antigenic relationships, which frequently occurred in slide agglutination could always be demonstrated also by tube agglutination with boiled cultures. These examinations which showed the numerous cross reactions, served as a base in the composition of polyvalent O sera.

Sera for typing according to KAUFFMANN and PERCH's simplified schema could be used unabsorbed. The following O sera, however, had to be absorbed

Serum	0 : 26/strain		F458/absorbed	by	strain	XK
„	0 : 33/	„	U510/	„	„	F409
„	0 : 35/	„	F335/	„	„	F92
„	0 : 41/	„	F409/	„	„	U510
„	0 : 42/	„	F295b/	„	„	F110
„	0 : 43/	„	P513/	„	„	F16
„	0 : 48/	„	P368/	„	„	M205

Results

1. Comparison of media

Comparative studies were made to find the culture medium most suitable for isolation of Proteus and related organisms from faeces. Results are shown in Table I.

LOVREKOVICH's bismuth sulfite medium seems to have definite advantages over the two other media as it supports the growth not only of Salmonellae but also of Proteus. Both of these form characteristic colonies, which, however, can easily be distinguished from each other. The superiority of Bi agar over the two other media is clearly shown by the fact that whenever Proteus was cultured from a faecal sample on ENDO's or D. C. media, growth could always be seen on Bi agar as well. Proteus strains isolated from non-faecal material on simple agar also grow readily on this medium. D. C. agar is somewhat more inhibitory to Proteus, and ENDO's agar yields positive results only if a great number of Proteus is present in the faeces. As the latter medium does not

inhibit coli bacteria, it is possible to estimate the number of *E. coli* and Proteus colonies. Non-selective or non-differential media are not recommended for the isolation of Proteus from faeces, as only strains swarming over the surface of the agar will be recognized this way.

Table I

Comparison of media

Culture medium	Of 833 faeces from infants	Of 1000 faeces from adults
	positive for Proteus	
Endo's.....	209	11
D. C.	337	30
Bi.	368	37
No. of positive	368	37

2. Results of faecal examination

Table II summarizes the incidence of Proteus bacteria according to examination groups. In the first group, stools of infants with diarrhoea were examined. In this group Proteus strains were isolated only on ENDO's agar. In the second group other cases of infantile diarrhoea were studied, while the third group included normal infants. Faeces of the fourth examination group were obtained from nurseries where a considerable number of infants were suffering from diarrhoea. For the isolation of Proteus in groups 2, 3 and 4, three media were used. All infants belonging to examination groups 1 to 4 were under one year of age. The fifth group included sporadic cases of suspected dysentery. Among these patients every age-group over one year was represented. In examination group 6 faeces from normal adults were studied. Groups 1, 2 and 4 included a few repeated examinations; each material in groups 3, 5 and 6 was collected from different individuals.

As from each faecal sample generally only one Proteus colony has been examined, the number of strains indicated in the Tables also means the number of positive stools.

Table II shows a striking difference in the incidence of Proteus in the various examination groups. A direct comparison of group 1 with the other groups cannot be made because of the difference in the media used. The considerable differences between examination groups 1 and 2 — particularly regarding the proportion of *P. morganii* strains — are due to the use of two more effective media in the latter. No satisfactory conclusions can be drawn from examination group 3 because of the small number of infants examined.

Table II

Proportion of isolations of Proteus bacteria, according to examination groups

Examination group	Culture medium used	Number of faeces examined	Faeces positive for Proteus		P. hauseri		P. morganii
			No.	%	No. of strains	No. of types at least	No. of strains
1. Infants with diarrhoea	E	4000	691	*17,2*	675	66	16
2. Infants with diarrhoea	E, DC, Bi	585	258	*46,2*	205	38	53
3. Normal infants	E, DC, Bi	102	16	*15,6*	13	8	3
4. Other infants	E, DC, Bi	248	110	*44,3*	100	29	10
5. Patients with suspected dysentery	DC	1035	119	*11,4*	52	24	67
6. Normal adults	DC, Bi	7419	249	*3,4*	197	71	52
Total	—	13,389	1443	—	1242	—	201

Abbreviations. E = Endo's agar, DC = desoxycholate citrate agar, Bi = bismuth sulfite agar.

It is remarkable that from normal adults Proteus was cultured only in 3,4 per cent, by the same technique, however, the examination of infants with diarrhoea yielded a positive result in 46,2 per cent. From faeces of patients with suspected dysentery, Proteus was cultured more often than from those of normal adults. In comparing these results it should be noted that of the 119 Proteus cultures indicated in examination group 5, 36 were isolated from children under 5 years of age.

Table III

Frequency of different enteric pathogens in faeces from infants with enteritis

Total number of faeces examined : 4585		
	No.	per cent
E. coli 111 : B4	642	14,0
„ 55 : B5	62	1,3
„ 26 : B6	38	0,8
„ 86 : B7	30	0,6
Shigella sonnei	24	0,5
„ flexneri	7	0,1
„ boydii	1	0,02
Salm. typhi murium	1	0,02
„ kottbus	1	0,02

Isolation of pathogenic enteric bacteria were also recorded. In Table III figures are given for the frequency of isolation of enteric pathogens from the faeces of infants with diarrhoea.

Among normal infants *E. coli* 111 : B 4 was cultured from 8 out of 102 samples. In group 5 the faeces of 15,2 per cent of the patients were positive for Shigella. It should be noted that in this group there were a great number of patients with simple diarrhoea not caused by Shigella.

Table IV shows the result of the serological and biochemical analysis of 1242 *P. hauseri* strains. Studies were made as to the frequency of *P. hauseri* types in the various examination groups. Table V shows the distribution of strains belonging to the most frequently occurring O antigenic groups.

In the faeces from cases of infantile diarrhoea three O groups emerged, namely O : 3, O : 26 and O : 13. The most frequently occurring strains in group 4 also belonged to these serological groups. From Table V it is clear that the incidence of these O groups in the faeces of normal adults was not more frequent than of other types. In group 5 the frequency of O : 3 strains was not so high as in infants with diarrhoea, but nevertheless this O group was more common than the other groups. It should be emphasized, however, that of the 13 O : 3 strains 11 were isolated from patients under two years of age.

Strains from infantile enteritis seem to be serologically more homogeneous than those from normal adults. Of the former strains 84,0 per cent, of those from normal material only 50,8 per cent could be typed.

The results given in Table V, showing the frequency of certain serotypes in cases of infantile enteritis, had, however, to be studied further, since a certain Proteus serotype, as a harmless inhabitant of the intestinal system might have been present in the majority of those infants who were being treated in one and the same hospital. To be certain that the distribution of strains belonging to the frequently occurring O groups is not varying from place to place, Table VI has been prepared from the data obtained in group 2.

From Table VI it is clear that strains belonging to the group O : 3 were the ones most often found in all hospitals but one. O : 3 strains also were the commonest types among strains from infantile diarrhoea of other origin. Materials from these latter cases were sent in by at least 30 different physicians and smaller hospitals. Strains belonging to O group 26 were frequently met with in Hospital 1. This high incidence seems to be quite out of proportion as compared to figures shown by the other hospitals. The frequency of Proteus O : 26 strains in group 1 may be explained by the fact that faeces from Hospital I have formed a considerable part of the material examined.

In cases of infantile diarrhoea one is liable to recognize an organism of potential pathogenicity as the causative agent, if it is present in the faeces in large numbers or in seemingly pure culture. Table VII shows the distribution of the most frequent *P. hauseri* O groups isolated in pure culture or in large

Table IV

List of the isolated P. hauseri types

Serotype	Bio-chemical type	No. of strains	Serotype	Bio-chemical type	No. of strains	Serotype	Bio-chemical type	No. of strains
			15 : 3	2	3	32 : 1	1	2
1 : 1	1	3	16 : ND	1	2	*32 : 6*	*1*	2
1 : ND	1	3	19 : 1	1	5	*33 : 2*	*2*	6
3 : —	2	59	*19 : 5*	*1*	1	*34 : 1*	*2*	1
3 : 1	2	257	*19 : 6*	*1*	2	34 : 6	1	2
3 : 2	2	28	19 : ND	1	1	*34 : 8*	*1*	1
3 : ND	1	3	20 : 1	2	1	34 : ND	1	2
4 : 1	1	4	20 : 2	2	2	*36 : 6*	*1*	1
4 : 8	1	2	21 : 1	1	4	38 : 2	2	3
4 : ND	1	1	22 : ND	1	2	39 : 18	1	2
5 : 1	2	6	*23 : 1*	*1*	1	*40 : 2*	*2*	1
5 : 2	*2*	1	23 : 1	2	1	41 : 2	2	1
6 : 1	2	3	23 : 2	2	3	*41 : 4*	*2*	4
6 : 3	2	40	23 : 3	2	4	*41 : 17*	*1*	1
6 : 4	*2*	1	23 : ND	1	2	42 : 1	1	1
7 : 1	2	1	23 : ND	2	3	43 : 2	2	1
7 : 6	*2*	1	24 : 1	2	5	43 : ND	1	1
9 : 1	2	1	24 : 3	2	3	45 : 11	1	1
9 : 2	2	5	24 : 13	2	4	45 : ND	1	2
9 : ND	1	1	25 : 1	1	3	47 : 1	1	1
9 : ND	2	1	25 : ND	1	1	ND : 1	1	12
10 : 1	2	28	26 : 3	2	180	ND : 1	2	30
10 : 2	2	10	26 : 6	1	7	ND : 2	2	76
10 : 3	2	8	26 : ND	1	13	ND : 3	2	31
10 : 4	2	2	*27 : 1*	*2*	3	ND : 4	2	1
10 : 5	2	2	27 . 2	2	3	ND : 5	1	1
11 : 2	2	7	27 : ND	1	1	ND : 5	2	1
12 : 1	1	1	28 : 2	2	20	ND : 6	1	16
12 : 8	*1*	1	28 : 3	2	5	ND : 8	1	3
12 : ND	1	4	*28 : 6*	*2*	1	ND : 13	2	1
13 : 1	2	83	28 : ND	2	2	ND : 15	2	1
13 : 2	2	16	29 : 13	2	3	ND : 17	1	1
13 : 3	2	14	30 : 1	2	7	ND : ND	1	70
13 : 5	*1*	1	30 : 2	2	5	ND : ND	2	20
13 : ND	1	1	31 : 1	1	2	Unstable	1	2
13 : ND	2	3	31 : 2	2	26	in saline	2	5
14 : ND	1	1	31 : ND	1	1			

Key : Serotype : Numbers ahead of colon mean the 0 antigens, those after colon the H antigens. Biochemical type 1= P. vulgaris, 2 = P. mirabilis. Types printed in italics are new ones. ND = new antigens, not determined so far.

numbers from 4585 faeces from patients with infantile enteritis. In obtaining these data, ENDO's medium was used. "Pure culture" means that no colonies of *E. coli* were observed on the plates ; if 50 per cent or more of the colonies proved to be Proteus, this was regarded as being present in large numbers. From Table VII it is evident that Proteus bacteria found in pure culture or in large numbers belonged to group O : 3 in most cases ; O : 26 group was also frequent. It should be noted that such great numbers of Proteus very rarely occurred in the faeces of normal individuals.

Table V

Distribution of the most frequently occurring P. hauseri O groups, according to examination groups
(Total number of strains, 1242)

O groups	1	2	3	4	5	6
	Infants with diarrhoea		Normal infants	Other infants	Patients with suspected dysentery	Normal adults
Total number of faeces	4000	585	102	248	1035	7419
0 : 3 ..	198	85	—	46	13	6
0 : 6 ..	32	4	—	1	2	6
0 : 9 ..	1	—	—	—	1	6
0 : 10 .	27	7	—	1	—	15
0 : 13 .	75	13	1	12	2	15
0 : 24 .	11	—	—	—	—	1
0 : 26 .	145	36	—	10	5	5
0 : 28 .	14	4	3	1	—	7
0 : 30 .	7	2	—	—	—	3
0 : 31 .	23	1	—	—	2	4
Other groups	142	53	9	29	27	129

Table VI

Incidence of the most frequently occurring P. hauseri O groups in various hospitals

Designation of hospital	I	II	III	IV	V	Other
Number of stools	114	97	42	73	55	204
O : 3	17 (16)	16 (10)	3	18 (15)	8 (7)	23
O : 10	1	—	—	—	—	6
O : 13	3	3 (2)	—	1	—	6
O : 26	24 (18)	1	1	5 (4)	1	4
Other O groups ..	13 (11)	7	4	8	5	27 (26)

Bracketed figures indicate the number of cases.

3. Result of serological examination of strains

As shown in Table IV, at least 107 different serotypes have been found among the 1242 *P. hauseri* strains. The real number of the serotypes is probably much greater, since non-determined (ND) antigens may represent a large scale of entirely different antigens. Of 1242 *P. hauseri* strains, 887, i. e. 71,5 per cent, could be fitted into types included in the KAUFFMANN—PERCH schema.

33 strains (2,6 per cent) fell into 19 still undescribed types, which, however, could be identified with sera of the KAUFFMANN—PERCH schema, as they repre-

sented new combinations of known O and H antigens. These new types have not been analysed in detail since the antigens had not been determined in cross-absorption tests. 315 strains contained new O and/or H antigens. Of the strains examined, 7 could not be determined serologically because they agglutinated spontaneously in normal saline. 6 strains gave a strong reaction in two different O sera. The latter strains were included in the number of strains with new, nonidentified antigens.

Table VII

O-group distribution of P. hauseri strains found in great numbers in faeces from infants with enteritis

O group	P. hauseri	
	in pure culture	in great numbers
O : 3	34	43
O : 6	3	6
O : 10	3	5
O : 13	9	21
O : 26	20	31
Other groups	9	40

59 strains were non-motile and all of them belonged to O group 3. Not a single strain which would have given a strong reaction in two H sera, nor phase variation was observed.

4. Result of biochemical examination of strains

Table VIII summarizes the biochemical behaviour of 1443 Proteus strains. It shows that the overwhelming majority of strains gave typical reactions.

21 strains could not be classified as either *P. hauseri* or *morganii*. 6 of them proved to be typical Providencia strains. Most of the remaining strains did not produce indole, but otherwise resembled Providencia cultures. No mannitol fermenting Proteus strains were isolated.

Discussion

Serological examination of *P. hauseri* strains especially from pathological material yielded a great percentage of cultures that could be typed. This fact shows the practical value of the KAUFFMANN—PERCH schema.

The result of examination of test strains of the KAUFFMANN—PERCH schema and of freshly isolated cultures, shows the diagnostic value of our O agglutination method. Provided that adequate controls are used, this technique affords a reliable and most rapid method of grouping a Proteus strain.

Table VIII

Biochemical behaviour of 1443 Proteus strains

	P. vulgaris	P. mirabilis	P. morganii
Urea	197 +	1043 +, 2 —	201 +
H_2S	197 +	1017 +, 28 —	6 +, 195 —
Indole	192 +, 5 —	4 +, 1041 —	201 +
Mannitol	197 —	1045 —	201 —
Maltose	195 +, 2 —	1045 —	1 $+^1$ 200 —
Sucrose	197 +	1041 ×, 4 —	17 ×, 184 —
Xylose	195 +, 2 —	1002+, 29 $+^1$, 14 —	2 $+^1$, 199 —
Ammonium citrate	—	—	1 +, 200 —

Abbreviations + = positive after 1 day
$+^1$ = positive after 2 to 13 days
× = weakly positive after 2 to 13 days
— = negative after 14 days.

Some conclusions of interest can be drawn by comparing the *P. hauseri* strains shown in Table IV with those isolated by PERCH. It is remarkable that cultures belonging to a certain serotype exhibit a similar biochemical behaviour *E. g.*, cultures of O antigen groups 1, 4, 21, etc. were members of biochemical type 1 *(P. vulgaris)*, according to both PERCH's and our examinations. O : 10, 20, 24, etc. strains, on the contrary, were members of the *P. mirabilis* group, according to both examinations. In some of the O groups strains belonging to both biochemical types were represented. In this case cultural properties were often characterized by the H antigen. *E. g.* serotype 26 : 3 always belonged to *P. mirabilis*, while 26 : 6 strains belonged to *P. vulgaris*, both in PERCH's and in the present investigations.

The remarkable similarity of the properties of Danish and Hungarian strains makes it very probable that there is a close connection between antigenic structure and biochemical behaviour.

*

From the present investigations it is clear that among infants suffering from diarrhoea some Proteus O groups, especially group O : 3, were much more frequent than others. O : 3 group strains were also frequently found as predominant organisms in the faeces of such infants.

One should, however, be very cautious, in recognizing a certain Proteus serotype as the aetiological agent of infantile diarrhoea. There is no proof that any of the serotypes were capable of initiating epidemic disease among infants, as pathogenic *E. coli* types do. No data were obtained from the present investigations as to the influence of antibiotic treatment on the occurrence of Proteus in the intestinal flora.

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Summary

A new slide agglutionation method for the determination of O antigens of *Proteus hauseri* strains has been described. Comparative studies have shown the bismuth sulfite medium to be the most suited for isolation of Proteus from faeces.

The serological and biochemical characters of 1242 *P. hauseri* strains isolated from 13 389 faecal samples has been described. The biochemical behaviour of 201 *P. morganii* strains has been reported. Of the *P. hauseri* strains 71,5 per cent could be typed according to KAUFFMANN and PERCH's antigenic schema. Strains belonging to O group 3 showed a remarkably high frequency among infants with diarrhoea, while the incidence of these strains among normal adults was not more frequent than that of other strains. The connection between antigenic and biochemical properties of the isolated strains was surprisingly similar to that of strains examined by PERCH.

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СЕРОЛОГИЧЕСКАЯ КЛАССИФИКАЦИЯ ТИПОВ ШТАММОВ, ПРОИСХОДЯЩИХ ИЗ СЛУЧАЕВ ЭНТЕРИТА ГРУДНЫХ ДЕТЕЙ И ДРУГИХ ИСТОЧНИКОВ

Б. ЛАНЬИ

Резюме

Автор описывает новый метод агглютинации на предметном стекле для определения О антигена штаммов P. hauseri. Согласно сравнительным исследованиям наиболее пригодной для выделения штаммов Proteus из испражнений является питательная среда из сульфита висмута. Описывает далее серологические и биохимические свойства 1242 штаммов P. hauseri, изолированных из 13389 проб кала. Касательно штаммов P. morgani сообщает только биохимические свойства.

Из штаммов P. hauseri 71,5% могли быть классифицированы по антигенной схеме Кауффмана—Перха. У младенцев, страдающих поносом, чрезвычайно часто обнаруживались штаммы, относящиеся к группе 0 : 3, в то время как встречаемость упомянутых штаммов в нормальном материале не была чаще встречаемости других штаммов. Зависимость между антигенными и биохимическими свойствами выделенных штаммов была поразительно подобна таковой штаммов, исследованных Перхом.

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